EXERCISE IS NEUROPROTECTIVE TO MOTONEURON DENDRITES FOLLOWING PARTIAL MOTONEURON DEPLETION VIA A MECHANISM DEPENDENT ON ANDROGEN RECEPTORS AT THE TARGET MUSCLE

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"The true conquests, the only ones that cause no regrets, are over ignorance"

Napoleon Bonaparte

Cory Charles Chew

EXERCISE IS NEUROPROTECTIVE TO MOTONEURON DENDRITES FOLLOWING PARTIAL MOTONEURON DEPLETION VIA A MECHANISM DEPENDENT ON ANDROGEN RECEPTORS AT THE TARGET MUSCLE

Motoneuron loss is a severe medical problem that can result in loss of motor control and eventually death. It has been previously demonstrated that partial motoneuron loss can result in dendritic atrophy and functional deficits in nearby surviving motoneurons, and that treatment with testosterone attenuates these structural and functional deficits. In this dissertation, I establish that exercise following partial motoneuron depletion is similarly neuroprotective to motoneurons, via a mechanism that requires androgen receptor activation at the target muscle of the affected motoneurons. I also establish that exercise transiently upregulates both circulating concentrations of testosterone and the density of androgen receptors in quadriceps musculature. Finally, I demonstrate that prior training with exercise is not able to confer resilience to subsequent neural injury, but that exercise may confer androgen sensitivity to a previously insensitive neuromuscular system. The findings in this dissertation demonstrate that exercise is neuroprotective to motoneurons via the same mechanism of androgen receptor activation in the target muscle seen with supplemental hormone treatment, and that a behavioral intervention such as exercise can have modulatory effects on both peripheral and central aspects of the neuromuscular complex.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

Neurons respond to insult

Neurodegenerative diseases and neural injuries can result in the loss of spinal motoneurons, potentially leading to compromised motor function, quality of life, or more severe pathologies. For example, amyotrophic lateral sclerosis (ALS) is the most common human motoneuron disease, affecting 2-3 people per 100,000. ALS pathology manifests in the progressive death of upper and lower motoneurons in the brain and spinal cord. As the motoneurons innervating muscles die off, the denervation causes the muscles to atrophy, resulting in weakness, loss of voluntary motor control, and eventually death, most commonly due to an inability of the respiratory muscles to induce breathing (Cleveland & Rothstein, 2001). ALS symptoms are hallmarks of the dangers of neurodegeneration, but neurodegeneration is not exclusive to motoneurons. Alzheimer's, Huntington's, and Parkinson's diseases are all neurodegenerative, each manifesting with different collections of symptoms and causes.

Diseases are not the only set of circumstances under which motoneurons can be damaged. Injuries to either the peripheral or central nervous system via automobile accidents, stroke, combat injury, traumatic brain injury (TBI), or a variety of other causes can result in permanent long-term damage (Dobkin, 1993; Menorca et al., 2013). Injured motoneurons that survive also show a variety of morphological and functional changes after such insults, often exacerbating the negative effects due to direct loss of motoneurons. For example, motoneurons undergo dendritic retraction and atrophy after injury (e.g., after spinal cord injury; Byers et al., 2012; Liu, 2014). Similarly, after peripheral axotomy motoneurons show functional and biochemical changes as well as dendritic atrophy (Sumner and

Watson, 1971; Titmus and Faber, 1990; Bisby and Tetzlaff, 1992; Kinderman and Jones, 1994; O'Hanlon and Lowrie, 1995; Yang et al., 2004).

The atrophy of the dendritic arbor following injury is an important change in neuronal morphology that has rippling consequences. The dendritic arbor is a critical characteristic of many types of neurons, making up a vast majority of neuronal membrane surface area and acting as the point of contact for the majority of synaptic connections (McLaughlin, 1972; Ulfhake and Kellerth, 1981; Cameron et al., 1985). We can think of dendrites as forming much of the metaphorical hardware of the computer that is the nervous system, and the structure of the dendritic arbor will heavily influence how the neuron will function. For example, the morphologies of motoneuron dendritic arbors vary depending on whether the motoneuron in question innervates fast or slow-twitch muscle fibers (Cullheim et al., 1987), and the dendritic arbors of the cat triceps surae show robust postnatal morphological changes that parallel the maturation of locomotor function (Ulfhake and Cullheim, 1988a,b; Ulfhake, Cullheim, and Franson, 1988).

Atrophy of motoneuron dendritic arbors can have negative impacts on their electrophysiological properties and functionality due to a loss of available surface area to make synaptic connections (Lu et al., 2001; Grudt and Perl, 2002; Mentis et al., 2011). With fewer synaptic connections made due to the decreased availability of necessary surface area, the normal function of both individual motoneurons and synchronous firing of populations of motoneurons are disturbed (Little et al., 2009; Mentis et al., 2011). This core relationship between neuronal morphology and the resulting electrophysiological functionality is an absolutely critical principle in understanding the fundamental nature of neuroscience and, by extension, neuroplasticity. The function of any biological entity (e.g., cell, tissue, organ) is heavily influenced by its physical structure that determines how it will interact with other aspects of the physical world, and changes to that physical structure can produce changes to how said entity functions.

While the focus of this dissertation will be on motoneurons, it is important to bear in mind that injuries and disease affect many different subtypes of neurons that share common features and properties, which allow us to extrapolate some of our findings beyond the observed populations. For example, both hippocampal and amygdalar dendritic structures are modified following a moderate TBI (Gao et al., 2011; Hoffman et al., 2017). Interestingly, the two neuronal populations observed in these TBI studies showed contrasting alterations to their dendritic morphologies. Gao and colleagues (2011) observed fragmentation, less branching, and decreased synaptic stability in dendrites of mature granular cells in the hippocampal dentate gyrus, while Hoffman and colleagues (2017) observed robust increases in dendritic branching and complexity in both pyramidal and stellate cells of the basolateral amygdala.

These two examples raise a key question regarding the nature of how to interpret changes to neuronal structure: what makes a structural alteration 'good' or 'bad'? Gao et al. (2011) and Hoffman et al. (2017) observed opposite changes to dendritic structure in different regions following a similar injury, and it stands to reason that a TBI is not an event that will be a boon to proper neuronal function. But how can such a harmful event produce neuronal changes of opposite valence? The answer is unclear and this dissertation is not capable of providing a satisfying answer that is applicable to all models. However, the following is clear: while we may not currently be able to explain why some dendrites fragment after TBI and others hypertrophy following a similar injury with the available data, we can broadly conclude that regardless of the direction of change, dendritic remodeling can produce changes to the cell's ability to properly function.

Damage beyond the initial site

These negative functional and structural changes observed in motoneurons following injury are not limited to populations directly affected by the insult. Somatic motoneurons also show similar negative changes following insults to their neighboring cells. For example, in rats, motoneurons

innervating the vastus lateralis muscle of the quadriceps show atrophy of their dendritic arbors following the death of motoneurons innervating the adjacent vastus medialis despite no cell death observed in the population innervating the vastus lateralis (Figures 1.1, 1.2; Little et al., 2009). The motoneurons innervating the two muscles are located in a common motor pool (Figure 1.3), and the death of vastus medialis motoneurons produces negative changes in the vastus lateralis motoneuron population via some yet to be discovered mechanism. This will be referred to throughout this dissertation as either 'collateral atrophy' or 'secondary atrophy.' This collateral atrophy has been shown to not be driven by an influx of activated microglia, and current working hypotheses suggest that local changes to the neuropil following the death of vastus medialis motoneurons may cause the atrophy in vastus lateralis motoneurons (Chew et al., 2019). That neural injuries can affect the properties of seemingly unharmed cells makes understanding the mechanisms of both the cause of secondary atrophy and therapeutics that can mitigate the spread of damage of the utmost importance.

Therapeutics can attenuate both direct and secondary damage

There have been many different avenues of investigation in the development of therapeutics for neural injuries. Some fascinating exploratory ventures have used novel biotechnologies to provide structural and trophic support to regenerating axons (Ellis-Behnke et al., 2006). Others have focused on surgical advancements to repair existing damaged nerves and prevent degeneration from occurring at all (Ghergherehchi et al., 2019a,b). Many of these larger scale interventions have attempted to take advantage of the physiological responses induced by their interventions in order to promote recovery in the neuronal structures of interest, while biomolecular and/or pharmacological interventions have attempted to go straight to the source, as it were, of inducing the physiology of the organism to repair itself. Administration of exogenous neurotrophins, predominantly brain derived neurotrophic factor (BDNF), has been experimented with in a variety of injury models and delivery mechanisms, with

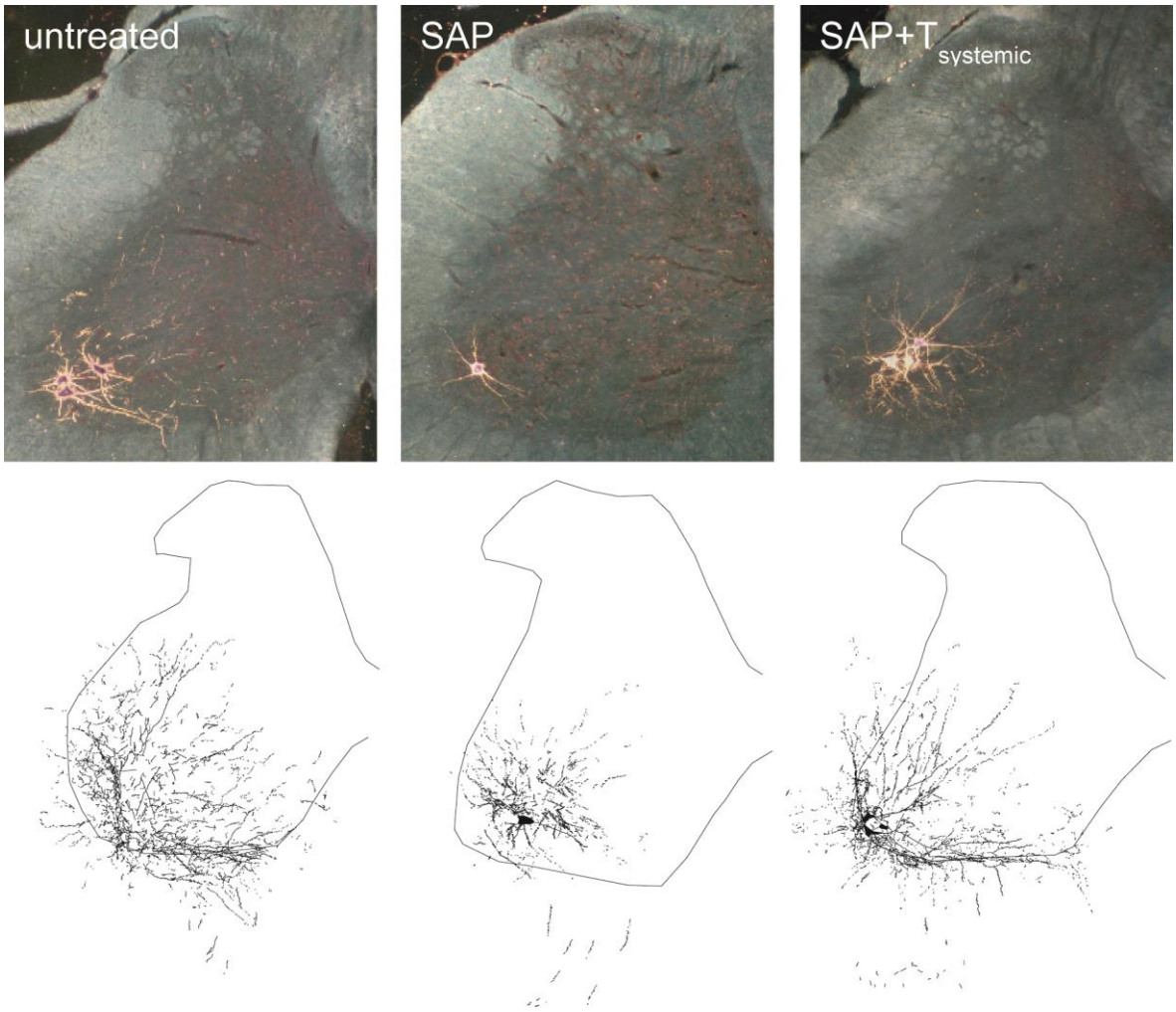

Figure 1.1. Darkfield digital micrographs of transverse hemisections through the lumbar spinal cords of untreated and saporin-injected animals with either no further treatment (SAP) or Silastic implants containing testosterone placed interscapularly (SAP + $T_{systemic}$). Saporin injection causes death of motoneurons in the injected musculature and atrophy in the dendrites of the surviving motoneurons, labeled here with BHRP. Treatment with testosterone attenuates the dendritic atrophy.

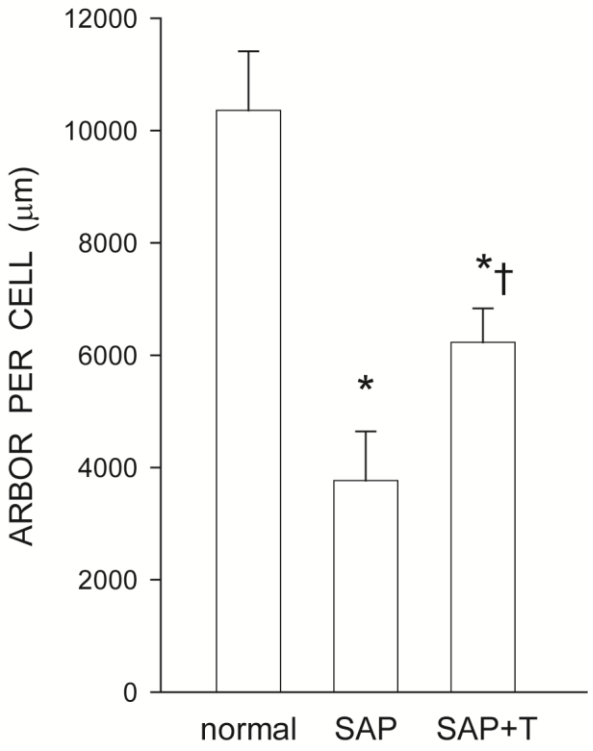

Figure 1.2. Dendritic lengths of quadriceps motoneurons in untreated animals and saporin-injected animals that either received no further treatment (SAP) or were given Silastic implants containing testosterone placed interscapularly (SAP+T). Following saporin-induced motoneuron death, surviving neighboring motoneurons lost almost 64% of their dendritic length and testosterone treatment attenuated this dendritic atrophy. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$). † indicates significantly different from untreated saporin-injected animals ($p < 0.05$).

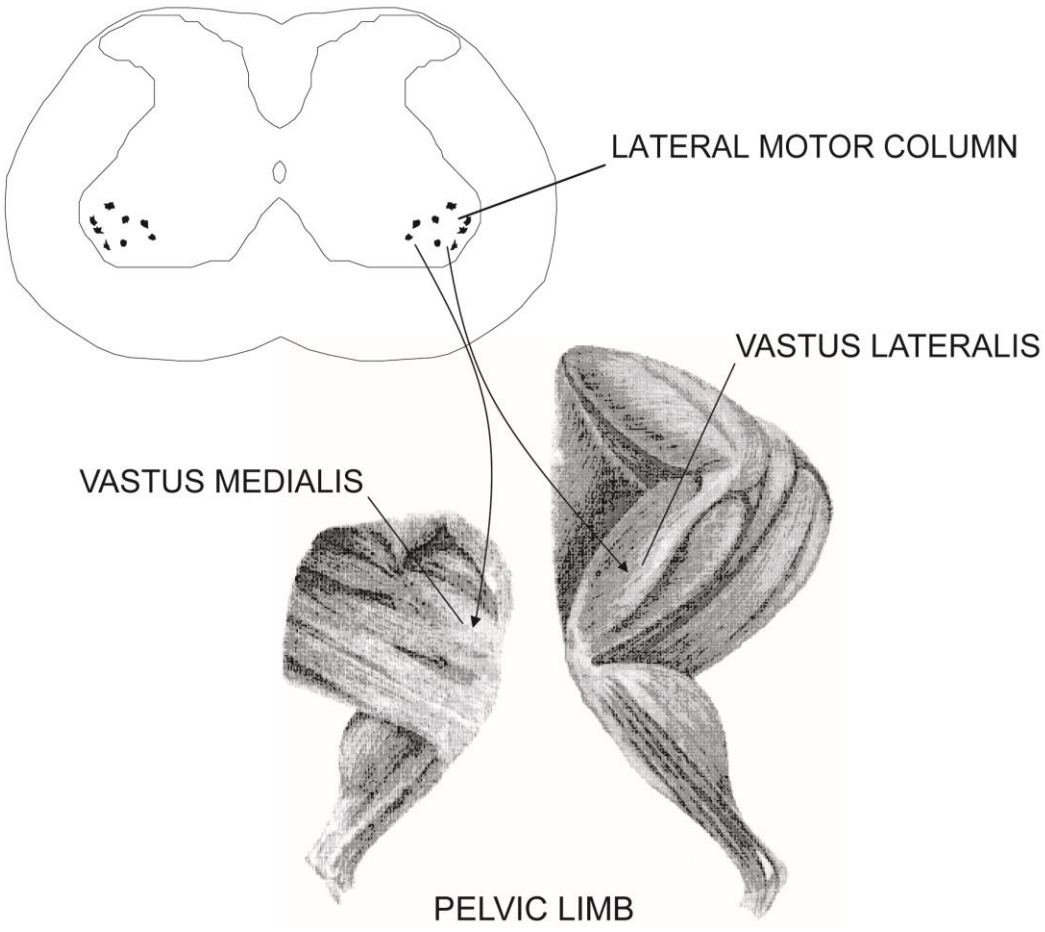

Figure 1.3. Diagram of the structure of the quadriceps muscle complex and innervating motoneurons in the lateral motor column of the lumbar spinal cord. Note that the motoneurons innervating the vastus lateralis and medialis muscles of the quadriceps are located in a common motor pool.

results typically suggesting that neurotrophins are effective in preventing or attenuating neuronal death and promoting neuroregeneration (Yang and Arnold, 2000; Liu et al., 2002; Yang et al., 2004). Importantly, it seems that specific neurotrophins and dosages are effective in treating some injuries and not others (Vejsada et al., 1994; Giehl and Tetzlaff., 1996; Bradbury et al., 1998). For example, Giehl et al. (1996) found that application of either BDNF or neurotrophin-3 (NT-3) was effective in preventing axotomy-induced death of corticospinal neurons, while nerve growth factor was not. Furthermore, while BDNF and NT-3 were both effective in preventing corticospinal neuron death, BNDF application was effective in preventing somal atrophy, while NT-3 had no effect on soma size (Giehl et al., 1996). Clearly, many variables contribute to the calculus of neuroprotective mechanisms.

Gonadal steroids also exhibit a wide variety of neurological benefits. For example, treatment with exogenous testosterone accelerates both axonal regeneration (Kujawa et al., 1991) and restoration of function (Kujawa et al., 1989) following facial motoneuron axotomy. This promotion of axonal regeneration has been shown to be dependent on many factors. First, the acceleration of axonal regeneration has been shown to be dependent on activation of the androgen receptor, as simultaneous treatment with testosterone and the androgen receptor antagonist flutamide fails to produce the acceleration of axonal regeneration (Kujawa et al., 1995). Second, the magnitude of the increased rate of acceleration is dependent on the temporal window in which testosterone is given; if testosterone treatment is delayed by 6 days, then there is no acceleration of functional recovery (Kujawa and Jones, 1990). Interestingly, if testosterone is given for the initial 7 days following crush axotomy and then ceased for the duration of recovery, behavioral recovery is still observed (Kujawa and Jones, 1990).

Each of these factors grants some new insight to the overarching mechanisms of how gonadal steroids are neurotherapeutic. The steroid receptor dependency of the neurotherapeutic effect suggests that there is a specific site in which the hormones must act in order to produce the desired effect, while

the temporal window of efficacy informs that there are critical periods following injury in which neurological systems are particularly susceptible to plastic modification, similar in concept to the critical periods observed during development in which brief exposure to hormones can have permanent organizational effects (Breedlove and Arnold, 1983a,b). While not definitive, the similarities between phenomena seen during development and successful treatment following injury suggest that there are common principles of neuroplasticity.

Our previous work has utilized a partial depletion model of neural injury to demonstrate that focal elimination of only some motoneurons can produce atrophy in the dendritic arbors of the surviving members of the same population (Fargo & Sengelaub, 2004a,b; Little et al., 2009). Much of this work has centered on the somatic motoneurons innervating the muscles of the quadriceps (Figure 1.3). The quadriceps is a muscular complex composed of four individual muscles: the rectus femoris, vastus medialis, vastus lateralis, and vastus intermedialis. These muscles are innervated by motoneurons projecting from the spinal cord via the femoral nerve, and the somata and dendritic arbors of these motoneurons make up a shared motor pool in the lateral motor column of the second lumbar segment of the spinal cord. Selectively killing motoneurons innervating a specific muscle of the quadriceps via intramuscular injection of the toxin saporin has allowed us to examine the effects of motoneuron death on the adjacent, surviving motoneurons of the shared quadriceps motor pool (Little et al., 2009; Cai et al., 2017; Chew et al., 2019).

Saporin is a ribosome inactivating protein derived from the soapwort plant (Stirpe et al., 1987). On its own, saporin is relatively benign; while the toxin is capable of irreversibly inactivating ribosomes and inducing cell death, it is not capable of crossing the cell membrane due to its lack of carbohydrate content. Conjugation of saporin to the appropriate subunit expressing the necessary surface molecules allows for selective entry to cells that express the conjugate surface molecules. Specifically, conjugation

of saporin to the cholera toxin B-subunit (CTB-saporin) allows for selective entry of saporin to cells expressing GM1 gangliosides, which is abundantly expressed in neurons (Abe and Norton, 1974). Thus, injection of CTB-saporin into the nerve terminal of the peripheral musculature allows for selective endocytosis of CTB-saporin by the innervating motoneuron, retrograde transport to the somata in the spinal cord, and eventual cell death in the days following injection (Fargo and Sengelaub, 2004a,b). This allows for induced death of only motoneurons innervating the CTB-saporin injected musculature and examination of the effects of that cell death on neighboring populations.

This secondary atrophy of surviving motoneuron dendrites can be attenuated via treatment with testosterone or other gonadal hormones (Fargo & Sengelaub, 2007; Little et al., 2009). Normally, the length of vastus lateralis dendrites will be reduced roughly 60% following the death of neighboring vastus medialis motoneurons However, if animals are given a subcutaneous implant filled with crystalline testosterone simultaneously with injection with CTB-saporin, dendritic length is reduced by only 40% (i.e., a 33% attenuation of dendritic atrophy; Figures 1.1, 1.2; Little et al., 2009). Furthermore, treatment with testosterone metabolites, estradiol or dihydrotestosterone, also attenuates dendritic atrophy to a comparable degree (Cai et al., 2017).

Mechanisms of hormone driven neuroprotection

The neuroprotective effects of gonadal steroid treatment against secondary atrophy seem to utilize a similar mechanism of action as seen in hormone treatment to primary neural injuries (e.g., nerves that have themselves been axotomized; Kujawa et al., 1991) Attenuation of quadriceps motoneuron dendrite atrophy following the death of their neighbors has also been shown to be dependent on steroid receptor action (Cai et al., 2017). As previously mentioned, treatment with testosterone following CTB-saporin injection is sufficient to attenuate dendritic atrophy (Little et al., 2009). Testosterone itself binds to the androgen receptor. However, testosterone can be metabolized into

either dihydrotestosterone or estradiol, an estrogen (Naftolin and MacLusky, 1982). This metabolic process makes it unclear whether any observed therapeutic benefits of testosterone treatment are due to activation of the androgen or estrogen family of receptors, and thus necessitates experiments utilizing implantation of either estradiol or dihydrotestosterone, an androgen that cannot be metabolized into an estrogen, to determine which family of receptors are responsible in providing neuroprotection. Cai et al. (2017) found that male rats injected with CTB-saporin simultaneously treated with either dihydrotestosterone or estradiol and antagonists for their cognate receptor (flutamide and tamoxifen, respectively) do not show attenuation of dendritic atrophy. This demonstrates two important phenomena: that both androgens and estrogens can be neuroprotective to motoneuron dendrites following the death of their neighbors, and that activation of their cognate receptor is necessary for both hormones to be neuroprotective.

That neuroprotective gonadal steroid treatment is steroid receptor dependent corroborates many phenomena that have also been observed in development and maintenance of the adult nervous system. Normally, the presence of androgens reduces normally occurring cell death seen during development of the masculinized spinal nucleus of the bulbocavernosus (SNB; Nordeen et al., 1985) and dorsolateral nucleus (DLN; Sengelaub and Arnold, 1989). SNB motoneurons proliferate equally in both sexes during normal development (Nordeen et al., 1985; Sengelaub et al., 1989). However, in males or androgen-treated females, the number of SNB motoneurons that undergo naturally occurring cell death is significantly lower compared to normal females at the appropriate critical period (Nordeen et al., 1985; Sengelaub et al., 1989). This effect was later found to be driven by androgen receptor activation at the target musculature of the SNB, the bulbocavernosus and levator ani muscles of the perineum (BC/LA; Fishman and Breedlove, 1992). The same androgen receptors in the SNB target musculature are also critical in the maintenance of those dendritic arbors in adulthood (Kurz et al., 1992; Rand and Breedlove, 1995). Development of SNB dendrites has also been found to be dependent on androgens

(Goldstein et al., 1990) and on the presence of the SNB target musculature (Goldstein et al., 1993), providing strong, if indirect, evidence that androgen action at the target muscle is key for normal SNB dendrite development. This proposed necessity of androgen action at the target muscle for proper neural development and maintenance of SNB dendrite structure in adulthood highlights an important mechanism that can be applied to hormonal neurotherapeutics following injury: that hormone action at the target muscle can alter innervating motoneuron structure.

Indeed, the efficacy of testosterone treatment in attenuating dendritic atrophy in quadriceps motoneurons following the death of their neighbors has also been found to be dependent on androgen receptor action at the target muscle (Chung, 2015). However the identification of the site of action raises interesting questions about a few key characteristics that pertain to the site in question. After all, muscles vary in their properties (e.g., striated vs. smooth, fast vs. slow twitch fibers, etc.), and there is a key difference in the muscular properties of the two neuromuscular systems observed in our model that is relevant to the mechanism of neuroprotection by gonadal hormones.

The perineal neuromuscular system is exceptionally androgen sensitive. Both the centrally located SNB and peripheral BC/LA accumulate radiolabeled androgens to a significantly greater degree than neighboring motoneurons of the same spinal segment or somatic skeletal muscle, respectively (Krieg et al., 1974; Breedlove and Arnold, 1980), and the BC/LA musculature expresses androgen receptors in quantities up to seven times higher than observed in somatic skeletal muscle (Krieg, 1976). This sensitivity to androgens has previously been discussed in this dissertation to affect the development and maintenance of the structure and function of this neuromuscular complex (Nordeen et al., 1985; Kurz et al., 1986; Goldstein et al., 1990, 1993; Rand and Breedlove, 1995).

This increased androgen sensitivity of the SNB-BC/LA complex raises an interesting question regarding the effectiveness of hormonal neurotherapeutics. After all, it has been established that the

neuroprotective effects of such treatments are dependent on steroid receptor action at the site of the target muscle (Chung, 2015; Cai et al., 2017), and the SNB-BC/LA complex expresses more androgen receptors at its target muscle. Thus, it stands to reason that the same hormonal treatment observed to be effective in attenuating dendritic atrophy in somatic quadriceps motoneurons following the death of their neighbors may be *more* effective in attenuating dendritic atrophy in androgen sensitive SNB motoneurons. This is, in fact, true. In the SNB, dendrites in testosterone-treated animals following CTB-saporin injection to the BC/LA are comparable to normal lengths (Fargo & Sengelaub, 2004b); in the quadriceps, vastus lateralis dendrites of testosterone treated animals following CTB-saporin injection to the vastus medialis are ~60% of normal length (Little et al., 2009).

The variation in neuroprotective magnitude in these two neuromuscular populations with differences in androgen sensitivity is not sufficient evidence to prove that the difference in the number of androgen receptors is responsible for the differences in neuroprotective magnitude. However, manipulation of the number of androgen receptors in the target muscle also has important modulatory effects on motoneuron properties that provide strong evidence that differences in androgen sensitivity can change how hormones affect motoneuron morphology. Normally, castration of adult male rats produces dendritic retraction in the motoneurons of the SNB, but not those innervating the quadriceps (Kurz et al., 1986; Huguenard et al., 2011). This difference in motoneuron morphology following castration is attributed to the highly androgen-sensitive nature of the SNB (Huguenard et al., 2011).

This hypothesis has been experimentally tested with the use of transgenic rats engineered to upregulate of the number of androgen receptors expressed in skeletal muscle. Normally, somatic skeletal muscles express androgen receptors at relatively low levels (Monks et al., 2004). However, transgenic rats with the human skeletal actin promoter (HSA-AR Tg) express 3.8x as many androgen receptors in the extensor digitorum longus compared to wildtype rats, and this phenomenon extends to other striated

muscles (Niel et al., 2009). When these transgenic animals are castrated, their quadriceps motoneurons show dendritic retraction that mirrors several of the changes seen in the SNB following castration; replacement of testosterone in castrates prevents this retraction (Huguenard et al., 2011). While this phenomenon does not directly indicate that experimental changes to target muscle androgen receptors can cause changes to neuroprotective magnitude of hormone treatments, it does indicate that changes to target androgen receptor expression can confer qualitative changes to innervating motoneuron populations that mirror naturally androgen sensitive populations. This opens the proverbial door to the notion that non-transgenic means of modulating androgen receptor expression may confer similar changes to motoneuron properties.

Exercise is neuroprotective

That the neuron-target tandem offers two potential sites for therapeutic intervention is also accompanied by the possibility that any modifications to one of the sites can have beneficial effects on the other. Exercise has been associated with strengthening of skeletal muscle since time immemorial, and in more recent decades, it has been strongly supported by the scientific community as beneficial for maintaining cognitive ability, emotional well-being, and physical health (Penedo & Dahn, 2005; Kramer et al., 2006; Hillman et al., 2008). Testosterone plays a well-documented role in adaptive changes following exercise. The classical dogma states that exercise increases testosterone output in order to build muscle mass (Bhasin et al., 1996, 2001a). However, further examination of the specific mechanism of action and time course of testosterone following exercise have led to some conflicting results and uncertainty as to how exercise and testosterone interact to produce the beneficial outcomes that are often observed.

Despite uncertainty surrounding the exact mechanism of the endocrine response following exercise, there is convincing evidence that hormonal responses are a necessary condition of many of the

benefits of exercise, including neuroprotective and neuroregenerative applications. Exercise has been implicated in upregulation of signaling factors that promote neuroplasticity (Vaynman & Gomez-Pinilla, 2005; Fouad & Tetzlaff, 2012) while also downregulating the expression of caspases associated with apoptosis following spinal cord injury (Sandrow-Feinberg & Houlé, 2015). Following axotomy, exercise increases both the number of regenerating axons sprouting from the proximal stump of the axotomized fibular nerve and the rate at which those axons regenerate, when compared to non-exercised animals (Sabatier et al., 2008). In the same axotomy model, treatment of exercised animals with the androgen receptor antagonist flutamide reduces the median length of regenerating axons to lengths comparable with animals who did not exercise (Thompson et al., 2014), suggesting that androgen receptor action is a necessary driver of the neuroprotective benefits of exercise following injury.

Given the neuroprotective role that exercise can have following axotomy, it is likely that exercise can have similar protective effects on motoneuron dendrites following induced atrophy. Data in Chapter 3 shows that rats allowed to run *ad libitum* after undergoing partial motoneuron depletion show dendritic lengths comparable to rats treated with testosterone implants (Figure 1.4; Chew and Sengelaub, 2019). This raises a number of questions concerning the mechanism of how exercise is protective to surviving motoneuron dendrites. Treatment with exogenous testosterone has been shown to depend on hormone action at the target musculature and is modulated by the hormonal sensitivity of those muscles. This similarity between the efficacy of exercise and exogenous testosterone as therapeutics, and the anabolic action of testosterone in adaptive effects following exercise suggest that any neuroprotective effects of exercise may utilize the same androgen receptor-driven mechanism seen in testosterone treatment.

Thus, the goal of this thesis is to investigate the following questions: 1) Is exercise neuroprotective for spinal motoneurons? 2) Does exercise utilize the same steroid receptor-driven neuroprotective mechanism as hormone therapies we have previously shown to be effective? 3) Does

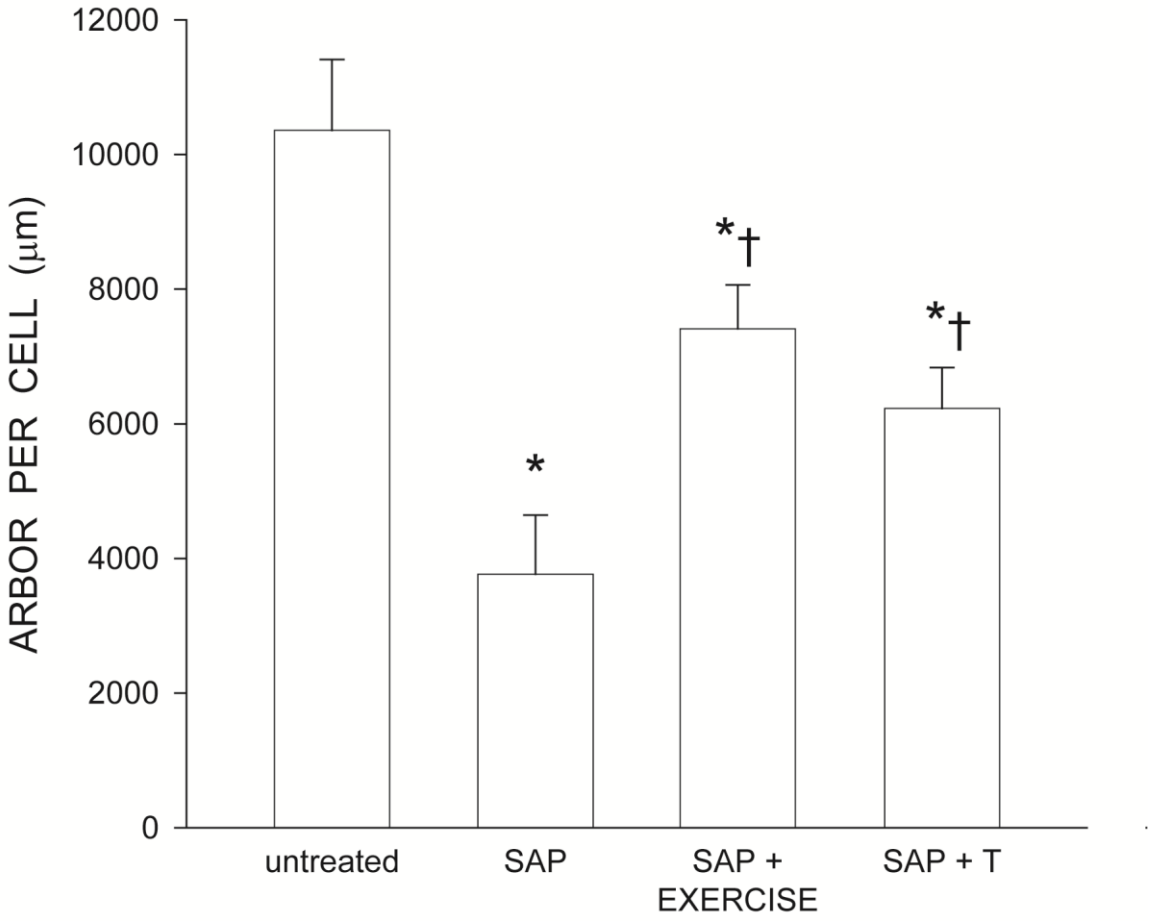

Figure 1.4. Dendritic lengths of quadriceps motoneurons in untreated animals and saporin-injected animals that either received no further treatment (SAP) or were allowed to exercise *ad lib* (SAP+EXERCISE). Data from Little et al. (2009) is included (SAP+T) to highlight the similarities in dendritic lengths in exercised saporin animals and saporin animals who are treated with testosterone. Following saporin-induced motoneuron death, surviving neighboring motoneurons lost almost 64% of their dendritic length and exercise attenuated this dendritic atrophy. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$). † indicates significantly different from untreated saporin-injected animals ($p < 0.05$).

exercise cause adaptive changes in skeletal musculature that facilitate any hormone-driven protective effects? 4) Can exercise confer resilience to subsequent neural injury?

Organization of the dissertation

This dissertation is divided into eight chapters. In this first chapter, I have provided what I believe to be necessary and relevant background information motivating the dissertation work. In the second chapter, I describe and justify the use of the methods and techniques used to address each of the questions of the dissertation. In chapters three through seven, I describe the motivating rationale, experimental design, results, and discuss the results of the individual experiments of the dissertation. In the eighth and final chapter of this thesis, I provide overarching commentary of how the findings of the entire dissertation coalesce and address yet-to-be-answered questions. Many of the experiments in this dissertation establish and expand upon a mechanism of exercise-driven neuroprotection, and the results of each experiment in isolation do not paint a complete picture. Thus, many of the individual chapter discussions will make allusions to other experiments, and the unified discussion in Chapter 8 will provide discussion of the mechanism in whole and how it may be interpreted.

CHAPTER 2

EXPERIMENTAL METHODS AND TECHNIQUES

In this chapter, I describe the methods and techniques used in the experiments of this thesis. Several of the methods and techniques are used in multiple experiments, and I will address the motivations and rationale for each technique here rather than reiterate their selection for each experiment. Any deviations or modifications of the methods and techniques described here will be addressed in subsequent individual chapters.

Animals

All animals were adult male Sprague Dawley rats purchased from Envigo (Indianapolis, IN), and were approximately 100 days old at the beginning of each experiment. Animals were maintained on a 12:12 light:dark cycle, with *ad libitum* access to food and water.

Rats who were allowed to exercise were given *ad libitum* access to exercise wheels (width = 11.2 cm; diameter = 37 cm; circumference = 116 cm) attached to their home cages. Wheel revolutions were tracked daily to ensure that rats were engaging in exercise throughout the time they were given access to a running wheel.

Saporin Injection

Saporin is a type 1 ribosome inactivating protein (RIP) derived from the soapwort plant (*saponaria officinalis;* Stirpe et al., 1987). Once saporin has crossed a cell's membrane, it acts an enzymatic catalyst to irreversibly inactivate ribosomes, halting protein synthesis and eventually leading to cell death (Stirpe, 2004). Saporin's classification as a type 1 RIP is due to its lack of an attached beta subunit, or B-chain. B-chains normally interact with lectins and other sugar molecules expressed on cell

membranes, and the lack of a B-chain prevents the enzymatically active saporin from entering cells. Conjugation of saporin to the appropriate B-chain expressing the necessary surface molecules allows for selective entry to cells that express the conjugate surface molecules. I have used saporin conjugated to the cholera toxin B-subunit (CTB-SAP; 2µL, 0.1% weight by volume; Advanced Targeting Systems, San Diego, CA) to allow for selective entry of saporin to cells expressing the GM_1 ganglioside, which is abundantly expressed in neurons (Abe and Norton, 1974). Thus, injection of CTB-SAP into the nerve terminal of the peripheral musculature allows for selective endocytosis of CTB-SAP by the innervating motoneurons, retrograde transport to the somata in the spinal cord, and eventual cell death in the days following injection (Fargo and Sengelaub, 2004a,b).

Previous use of this method has resulted in the successful induction of motoneuron death isolated to the motoneurons innervating the injected muscle. Unilateral injection to the BC/LA has shown death of 60% of the ipsilateral SNB motoneurons, while the contralateral SNB population was spared (Fargo and Sengelaub, 2004a,b). CTB-SAP injection to the left vastus medialis muscle of the quadriceps has shown a ~20% reduction in the number of motoneurons in the ipsilateral shared quadriceps motoneuron pool relative to the uninjected, contralateral side (Little et al., 2009). Because the vastus medialis is one of the four quadriceps muscles (rectus femoris, vastus lateralis, vastus intermedialis, and vastus medialis), it is expected that the percentage of motoneuron loss due to CTB-SAP injection to the vastus medialis is less than that seen in the SNB by injecting the BC/LA, in which surviving motoneurons innervate only the anal sphincter.

Animals were placed under general anesthesia by placement into an induction chamber with vaporized isoflurane. Animals were determined to be anesthetized by observation of their respiratory depth and rate. Animals were laid in the supine position, placed in a nose cone receiving vaporized isoflurane and oxygen in order to maintain sedation, and the upper portion of the left hind leg was

shaved and cleaned with iodine and isopropyl alcohol. The patella was palpated in order to determine the site of entry, and an incision was made vertically along the midline of the operated leg. The vastus medialis was exposed via blunt dissection and injected with 2µL of 0.1% CTB-SAP using a 10µL Hamilton syringe. The incision was then closed using stainless steel wound clips (MikRon Precision, Inc.; Gardena, CA) and the animal was placed in its home cage to recover from anesthesia.

Castration

Animals were placed under general anesthesia by placement into an induction chamber with vaporized isoflurane. Animals were considered to be anesthetized by observation of their respiratory depth and rate. Animals were laid in the supine position, placed in a nose cone receiving vaporized isoflurane and oxygen in order to maintain sedation, and a midline incision was made in the ventral scrotal wall. The tunica over the right testicle was grasped and pulled away from the testicle using forceps in order to cut a small opening in the tunica. The entire right testicle, epididymis, and as much of the testicular adipose tissue as safely possible were removed through this opening. The ductus deferens and blood vessels of the spermatic cord were ligated using silk suture before cutting the ductus and spermatic cord distal to the ligature. The ligated ductus deferens and spermatic cord were then replaced in the scrotum and the same process was repeated for removal of the left testicle. The scrotum was then closed using stainless steel wound clips, other surgical procedures were conducted as necessary, and a dose of the analgesic Meloxicam (2mg/mL; 0.1mL/100g body weight) was injected subcutaneously, and the animal was placed in its home cage to recover from anesthesia.

This castration procedure was effective in removing the body's major source of androgen production in males. While the adrenal gland does produce testosterone, it is a negligible amount compared to that of the testes. The removal of the testes serves to experimentally examine the effects of removing endogenous testosterone form the organism in question.

Hormone implants

Hormone implants were administered in the form of Silastic implants either filled or impregnated with crystalline hormones. Silastic silicon is a permeable material, allowing its contents to pass through it at a constant rate. Thus, passive diffusion and the resulting local concentration gradient dictate that the volume of hormone within the implant will dictate how much hormone is delivered to the recipient.

Testosterone implants

As previously mentioned, testosterone can be metabolized into estradiol, and estrogenic hormone, by the enzyme aromatase (Naftolin and MacLusky, 1982). Thus, it is not immediately clear whether any effects of testosterone treatment are androgenic or estrogenic in nature. However, testosterone was chosen as the hormonal treatment in this dissertation due to its use in the scientific literature motivating the design of these experiments (Fargo et al., 2004a,b; Little et al., 2009; Huguenard et al., 2011).

Testosterone implants were made by filling a length of Silastic silicone tubing (1.57mm inner diameter, 3.18mm outer diameter; Dow Corning; Midland, MI) with crystalline testosterone (4-androsten-17β-ol-3-one; Steraloids, Newport, RI). A 55mm length of Silastic tubing was cut and a 5mm wooden dowel was inserted into one end of the tubing. 45mm of the remaining tubing was filled with testosterone before another 5mm wooden dowel was inserted to the remaining tube opening. The implant was wiped clean with a Kimwipe (Kimberly-Clark, Neenah, WI) moistened with 100% ethanol to remove any external steroid residue before both ends of the tube were sealed with Silastic silicone medical adhesive (Dow Corning) and allowed to cure overnight.

Implantation took place under general anesthesia following saporin injection. After completion of prior surgical procedures, animals were laid prone, had a small area near the scapulae shaved and

cleaned with iodine and alcohol prior to incision. A small tissue pocket was made via blunt dissection and the implant was inserted interscapularly along the axial musculature. The incision was closed using stainless steel wound clips, and the animals were placed in their home cages to recover from anesthesia.

Serum concentrations of testosterone normally fluctuate throughout the day in healthy adult male rats (Smith et al., 1977), ranging from 0.3 – 8.9ng/mL plasma and averaging 2.36 ± 0.25ng/mL (Damassa et al., 1977; Södersten et al., 1983). 45mm implants such as these have been demonstrated to produce concentrations of testosterone in the bloodstream approximating the daily average titer (Smith et al., 1977).

Hydroxyflutamide implants

Hydroxyflutamide implants were made by mixing a crystalline form of the nonsteroidal androgen receptor antagonist hydroxyflutamide (0.2mg; 2-hydroxy-flutamide; LKT Laboratories, St. Paul, MN) into Silastic silicon medical adhesive (Dow Corning). Once thoroughly mixed, the Silastic adhesive impregnated with hydroxyflutamide was pressed into rectangular molds (12.5 x 3.5 x 1.5 mm) and allowed to cure. Once cured, the implants were removed from their moldings and sealed on five of their six faces with nail polish. By painting five of six faces of the implant, hydroxyflutamide is only able to be released from the unpainted face, allowing for local administration of the implant contents to a targeted region (Cai et al., 2017).

To investigate the effects of local androgen receptor blockade at the target muscle, some animals had hydroxyflutamide implants placed at the vastus lateralis muscle of the quadriceps. Implantation took place under general anesthesia immediately following saporin injection. The vastus lateralis muscle was identified and exposed via the same incision site over the midline of the left hind leg used to access the vastus medialis. Implants were sutured onto the left vastus lateralis with the unpainted face in contact

with the vastus lateralis. The incision was closed using stainless steel wound clips, and the animals were placed in their home cages to recover from anesthesia.

In order to account for possible systemic effects of hydroxyflutamide, some animals had hydroxyflutamide implants placed interscapularly, rather than at the vastus lateralis. After completion of prior surgical procedures, animals were laid prone, had a small area near the scapulae shaved and cleaned with iodine and alcohol prior to incision. A small tissue pocket was made via blunt dissection and the implant was inserted interscapularly along the axial musculature. The incision was closed using stainless steel wound clips, and the animals were placed in their home cages to recover from anesthesia.

Motoneuron visualization

Retrograde labeling

Vastus lateralis motoneurons were visualized using a retrograde label of horseradish peroxidase (HRP) conjugated to the B subunit of the cholera toxin. This conjugated form of HRP will be referred to as BHRP (Invitrogen, Carlsbad, CA). In the studies comprising this thesis, I use BHRP diluted in distilled water to 0.2% weight by volume solution. As previously discussed, the B subunit of the cholera toxin allows for the conjugated molecules to be endocytosed by cells expressing the GM_1 ganglioside, which includes nerve terminals of quadriceps motoneurons. Injection of BHRP into the peripheral musculature at the nerve terminal allows for endocytosis of BHRP into these motoneurons and retrograde transport to the somata in the spinal cord. Following the appropriate histochemical reaction (discussed below), BHRP labelling allows for visualization of the labeled somata and distal neurite branches (Fig. 2.1). This method has been shown to be a robust and reliable method of studying motoneuron anatomy and has been shown to be sensitive to changes in motoneuron structure due to hormonal manipulations (Kurz et al., 1986; Goldstein et al., 1990), spinal cord injury (Byers et al.,

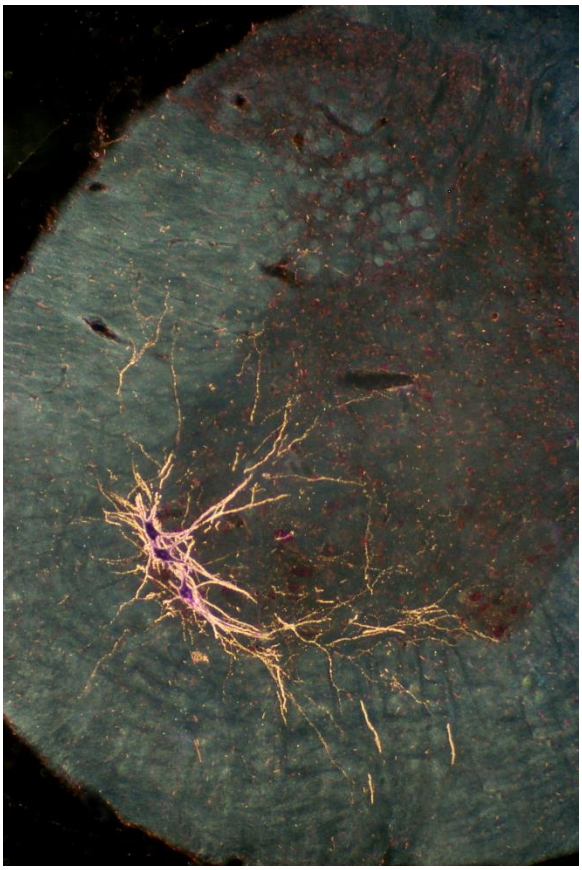

Figure 2.1 Darkfield digital micrograph of BHRP-labeled quadriceps motoneuron somata and neurites. BHRP labeling has been shown to be a robust and reliable method of studying motoneuron anatomy and has been shown to be sensitive to changes in motoneuron structure. Darkfield microscopy allows for increased contrast of the BHRP label against background, allowing for greater ease in dendritic reconstruction.

2012), death of neighboring motoneurons (Fargo and Sengelaub, 2004a,b; Little et al., 2009), and now behavioral interventions (Chew and Sengelaub, 2020). All studies examining neuronal structure in this thesis observe the same population of vastus lateralis motoneurons, so the procedure for injection of BHRP and labeling of motoneurons is the same across said studies.

BHRP injections were administered using a protocol similar to that of saporin injection, but with a few important distinctions. Animals were placed under general anesthesia by placement into an induction chamber with vaporized isoflurane. Animals were determined to be anesthetized by observation of their respiratory depth and rate. Animals were laid in the supine position, placed in a nose cone receiving vaporized isoflurane and oxygen in order to maintain sedation, and the upper portion of the left hind leg was shaved and cleaned with iodine and alcohol. The patella was palpated in order to determine the site of entry, and an incision was made vertically along the midline of the operated leg. The vastus lateralis was exposed via blunt dissection and injected with 2µL of 0.2% BHRP using a 10µL Hamilton syringe. The incision was then closed using stainless steel wound clips, and the animal was placed in its home cage to recover from anesthesia.

Prior studies have developed a reliable protocol and timeline for the use of BHRP as a retrograde label. These protocols have determined that two days is the optimal period of time for retrograde transport of BHRP from the injected quadriceps musculature to the somata and distal neurites within the spinal cord (Little et al., 2009; Cai et al., 2017). Thus, BHRP injections took place two days prior to sacrifice.

Spinal cord removal and processing

Two days after BHRP injection, animals were killed by an overdose of urethane. Animals were weighed and injected intraperitoneally with a lethal dose of urethane dissolved in physiological saline (0.25g urethane, or 0.5mL urethane solution, per 100g body weight). Euthanasia was assessed by

animals' failure to demonstrate righting reflex, response to toe pinch, or corneal reflex. If animals did not demonstrate areflexia within ~10 minutes, another 1mL injection of urethane solution was administered.

When animals were sufficiently anesthetized, they were placed in the supine position in order to allow access to the thoracic cavity in order to perform surgical procedures for secondary euthanasia and transcardial perfusion. The site of incision was located by palpating the sternum and making an incision in the skin just caudal to the sternum. Once the skin and upper layers of the abdominal musculature were cut, the xyphoid process was visualized through the translucent deeper layers of the abdominal musculature. The musculature was grasped with forceps just caudal to the xyphoid process and lifted away from the vital organs to ensure that the internal organs were not damaged as the abdominal wall was cut. A pneumothorax was then performed as a means of secondary euthanasia, the ribcage was cut on both the right and left sides, and reflected to expose the heart. The right atrium was cut and a cannula was inserted to the left ventricle. Animals were then transcardially perfused with cold physiological saline fed by a gravity pump until the perfusate ran clear (i.e., complete exsanguination), and was followed by perfusion with cold fixative (1% paraformaldehyde/1.25% glutaraldehyde; approximately 250mL).

Once perfused, animals were laid prone and an incision was made along the back over the vertebral column. The skin was spread and the axial musculature was excised, exposing the spinous processes. The most caudal rib was isolated via blunt dissection, and a laminectomy was performed approximately 1-2 cm rostral to the articulation point of the most caudal rib. The vertebrae were carefully removed until a 4 cm portion of the spinal cord was exposed. A portion of the spinal cord measuring 1cm from the articulation of the most caudal rib was removed and placed into cold fixative (1% paraformaldehyde/1.25% glutaraldehyde) for 5 hours, and cyroprotected in cold sucrose phosphate

buffer (10% weight by volume, pH 7.4) overnight. Spinal cords were then embedded in a gelatin block, and the block was fixed in a sucrose-glutaraldehyde solution diluted in phosphate buffer (10% sucrose weight by volume, 1.8% glutaraldehyde) for 18 hours, and cyroprotected in sucrose phosphate buffer (10% weight by volume, pH 7.4) for two hours.

Embedded spinal cords were frozen sectioned transversely at 40µm on a sliding microtome into four alternate series. One series was immediately mounted onto slides subbed with porcine gelatin, allowed to dry overnight, counterstained with thionin, and coverslipped with Permount. This unreacted series was used for cell counting, as the histochemical protocol used to visualize BHRP (described below) modifies pH of the tissue, resulting in understaining by thionin. The three remaining series were immediately processed with a histochemical protocol using tetramethyl benzidine as a chromagen in order to visualize BHRP for motoneuron morphological analyses (see below). Visualization of BHRP using this reaction results in labeled cells displaying a distinctive dark blue reaction product under brightfield microscopy (Fig. 2.2) that reflects as gold in color under darkfield microscopy (Fig. 2.1). Once the histochemical reaction was complete, these series were mounted, allowed to dry overnight, counterstained with thionin, and coverslipped with Permount.

Muscle dissections

Following the removal of the relevant portion of spinal cord, animals were flipped to the prone position. Incisions were made along the midline of the left hind leg and blunt dissection was used to expose the quadriceps muscular complex. The muscle origins cut, and the complex of the rectus femoris, vastus medialis, intermedialis, and lateralis was removed and dissected into its component parts. The vastus medialis and lateralis were weighed, and the process was repeated on the right leg.

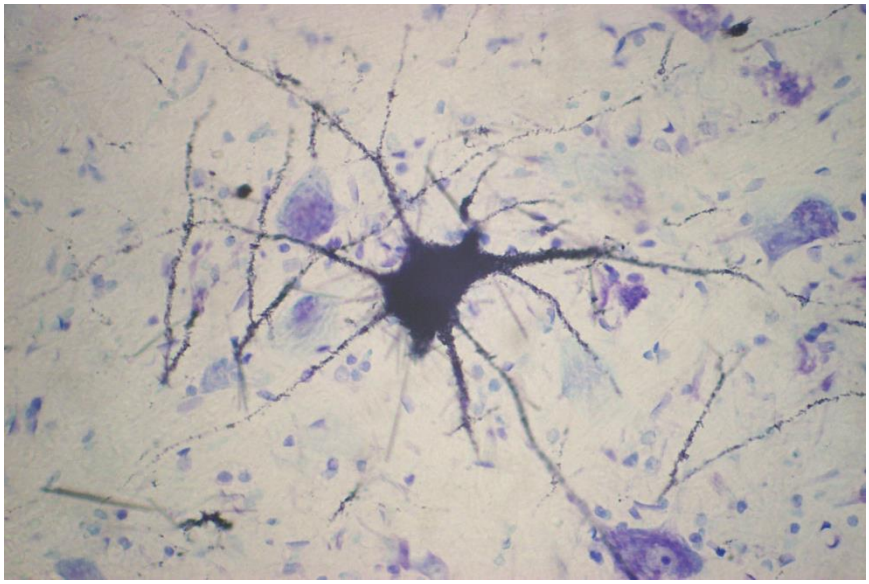

Figure 2.2 Brightfield digital micrograph of BHRP-labeled quadriceps motoneuron somata and neurites. In contrast with darkfield visualziation, BHRP reaction product appears as dark blue under brightfield microscopy. Darkfield microscopy limits the use of higher magnifcations, so brightfield microscopy was used for stereological counting as necessary.

In some experiments, effectiveness of hormonal manipulations (orchidectomy, androgen blockade, or testosterone treatment) was assessed by weighing the androgen-sensitive BC/LA muscles. To dissect these muscles, a midline incision was made over the scrotum, and the perineal muscles were exposed via blunt dissection. The penis, Cowper's glands, and adjacent ischiocavernosus were removed and the BC/LA was excised and weighed.

Microscopy

Motoneuron counts

Motoneurons are identifiable as large, multipolar cells that are darkly colored when counterstained with thionin (Fig. 2.3), and the observed quadriceps motoneurons are located in the lateral motor column of the second lumbar spinal segment. An optical disector method was used in Stereo Investigator (MBF Biosciences, Williston, VT) to obtain estimates of the total number of motoneurons in the left and right lateral motor columns. As previously mentioned, motoneurons were counted solely from the series of sections that did not undergo the BHRP visualization reaction.

For each animal, the range of sections in which BHRP-labeled motoneuron somata were present was identified, and corresponding sections from the unreacted series were used for counting. A counting frame (110 µm x 80 µm) was moved systematically throughout an area of each ventral horn (~500 µm x 500 µm) defined by previously established distribution of BHRP-labeled somata in each section within the identified rostrocaudal range. Motoneurons must meet all of the following stereological criteria to be counted: 1) there was a clearly defined nucleus and nucleolus, 2) they did not contact the forbidden lines of the counting frame, and 3) nucleoli must not appear in the first focal plane (i.e., "tops") as they appeared while focusing through the z-axis; tops were excluded to avoid double counting. The length of the disector was approximately 16 µm, which was adequate for visualizing nucleoli in multiple focal planes. Counts were made at x937.5 under brightfield illumination.

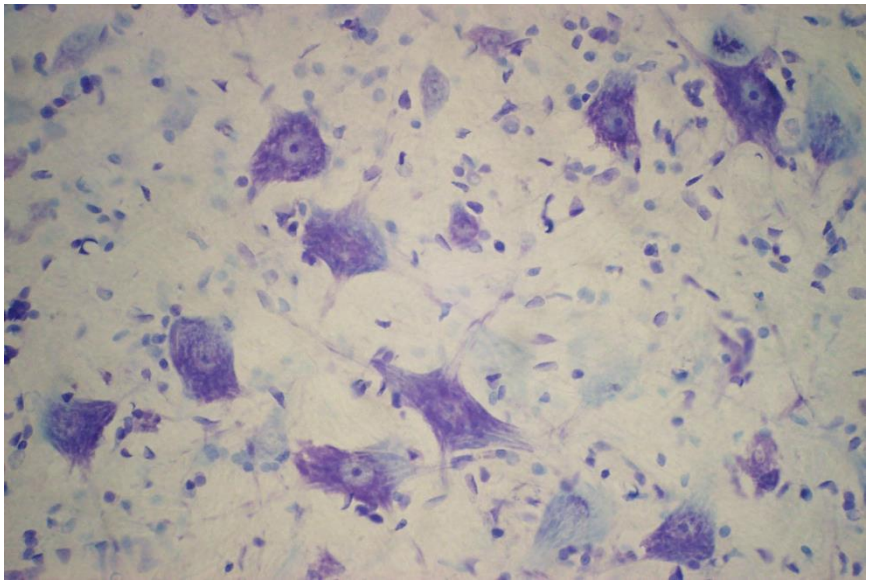

Figure 2.3. Brightfield digital micrograph of thionin-stained quadriceps motoneurons. Quadriceps motoneurons are large, darkly staining, multipolar cells. Following injection of saporin into the left vastus medialis, the number of motoneurons in the left quadriceps motor pool is severely depleted compared to the contralateral side. Cell counts of thionin-stained motoneuron are used to confirm saporin-induced motoneuron depletion.

Because only one of the four alternate series of sections was used for cell counts, the number of motoneurons counted was multiplied by four to correct for our sampling. Counts were then expressed as a ratio of motoneuron number on the saporin-injected (left) side relative to that on the untreated (right) side to quantify the magnitude of motoneuron depletion.

Motoneuron morphometry

Measures of motoneuron morphometry were collected from one of the three series that underwent histochemical processing (described above) to visualize BHRP label. Which series was determined by picking the series with the median number of BHRP labeled vastus lateralis motoneurons. In cases where two series had the same number of motoneurons, a qualitative judgment was made of the quality of the mounted sections.

Motoneurons labeled with BHRP were counted as described above for cell counts in the non-histochemically reacted series, but with a few important differences. Because the reaction product in BHRP labeled motoneurons often obscures the nucleus and nucleolus, BHRP-labeled motoneurons did not use the presence of a nucleus and nucleolus as counting criteria. Furthermore, this obfuscation of the nucleolus in BHRP-labeled motoneurons prevents the use of nucleoli in the first focal plane as an exclusionary criterion in stereological counting. Therefore, presence of BHRP reaction product in the first focal plane was used as an exclusionary criterion to prevent double counting.

Dendritic length. In order to assess the total dendritic length of the BHRP-labeled motoneurons, a computer-based morphometry system (Neurolucida, MBF Biosciences) with a Lucivid connected through a camera lucida tube was used to reconstruct dendritic arbors in three dimensions. Sections with BHRP label present were identified and catalogued to ensure that the entire rostrocaudal extent of the label was captured. Every third section (480μm apart) was traced under darkfield illumination at a final magnification of 250x. Tracings of individual sections along the rostrocaudal extent of the label were

then merged into a single composite, and the total dendritic length of this composite was calculated, multiplied by three to correct for sampling, and divided by the number of BHRP labeled motoneurons to determine the dendritic arbor length per labeled cell.

It is important to note that this measure does not represent a measurement of total dendritic length (Kurz et al., 1991). However, as previously mentioned, this method produces a reliable estimate of dendritic length that is capable of reflecting changes in dendritic length during normal development (Goldstein et al., 1990, 1993) and adulthood, including changes in dendritic length due to hormonal manipulations (Kurz et al., 1986; Goldstein et al., 1990) and induced motoneuron death (Fargo and Sengelaub, 2004a,b; Little et al., 2009). For convenience sake, this measure will be referred to interchangeably as either 'dendritic length' or 'arbor per cell.'

Dendritic distribution. A set of radial axes was overlaid on the reconstructed composite of each animal, with the central intersection of the axes centered on the center of the somata of BHRP-labeled motoneurons. These axes were divided into 12 radial bins of 30° each, and dendritic length per bin was determined by summing the lengths within that bin. This radial binning provides a measure of whether induced motoneuron death, exercise, or any of our other experimental manipulations cause or contribute to a reorganization of dendritic distribution.

Dendritic extent. The same set and position of radial axes described in the dendritic distribution were used to assess the extent of dendritic length in three dimensions. First, the linear distance between the center of the radial axes and the most distal BHRP-labeled process was calculated in order to determine the maximal extent of dendritic distribution in the transverse plane. Second, the rostrocaudal extent of the BHRP label was calculated by counting the number of sections in which BHRP label was present in the traced series. This number of sections was then multiplied by the known distance between sequential

sections of a given series (160µm) to determine the linear rostrocaudal extent of BHRP label. Together, these measures are able to determine the three dimensional extent of BHRP label in a given animal.

Testosterone assays

Blood samples were collected at the time of sacrifice via cardiac puncture. Following collection, blood samples were allowed to rest at room temperature for one hour to allow for clot formation. The clot was then removed, and samples centrifuged at 4°C for 30 minutes at 2500rpm. The sera was separated from the red blood cells by pipetting into fresh tubes and stored at -20°C until assays were run.

Assays for testosterone concentrations were run using an ELISA kit per the manufacturer's recommended specifications (ADI-900-065, Enzo Life Sciences, Farmingdale, NY). Sera samples were diluted 1:40 using the assay buffer provided by the kit manufacturer.

Androgen receptor immunohistochemistry

Androgen receptor expression in the vastus lateralis was assessed by fluorescent immunohistochemistry. In order to confirm the location of any labeled androgen receptors in the muscle, sections were co-labeled with fluorescent antibodies for the basal laminar membrane. The basal lamina is a membrane surrounding epithelial cells, and is known to surround muscle fibers (Monks et al., 2004; Rudolph and Sengelaub, 2013). By co-labeling tissue with fluorescent secondary antibodies for both androgen receptors and the basal lamina, I was able to quantify and compare the number of androgen receptors in the tissue, and determine whether or not any labeled androgen receptors were localized to muscle fibers.

Muscle dissections and cryosectioning

Following overdose with urethane using the method described above, animals underwent secondary euthanasia via pneumothorax, and the left vastus lateralis was fresh dissected in the same

fashion as described above. The vastus lateralis was then flash frozen in methylbutane chilled with dry ice, adhered to a chuck with M1 embedding matrix (Thermofisher), and placed in a cryostat previously chilled to -20°C for one hour to acclimate. Muscles were sectioned transversely at 12µm into three series, thaw mounted onto porcine gelatin subbed slides, and stored in opaque slide boxes. Two series were sealed in airtight bags and stored in reserve in a -80°C freezer; the primary series was sealed in an airtight bag in a -20°C freezer overnight.

Immunohistochemistry

Sections were removed from the -20ºC freezer and allowed to come to room temperature prior to opening of the sealed bag. Once temperature acclimated, sections were encircled with a lipophilic Super Pap Pen (Ted Pella Inc.), and the lipophilic barrier was allowed to air dry for five minutes. Sections were fixed for five minutes in a bath of 4% paraformaldehyde, rinsed in phosphate buffered saline (PBS), and incubated for one hour at room temperature in a blocking solution of 10% normal goat serum and triton-X in 0.01M PBS pipetted onto individual sections. Sections were then incubated 48 hours at 4°C in PG-21 rabbit anti-androgen receptor primary antibody (1:1000 dilution; 06-680, Millipore Sigma, Temecula, CA). One section on each slide was pipetted with a solution lacking anti-androgen receptor antibody as a negative control for androgen receptor labeling; this negative control section subsequently received AlexaFluor 488 secondary antibody, D18 primary anti-laminin, and TRITC secondary antibodies. After incubation, sections were rinsed in 0.01M PBS and incubated for two hours at room temperature in AlexaFluor 488 fluorescent goat anti-rabbit IgG [1:200; A-11070, Invitrogen, Eugene, OR) pipetted onto individual sections.

Immediately following incubation with the androgen receptor secondary antibody, sections were rinsed in 0.01M PBS and incubated overnight at 4ºC in D18 anti-laminin primary antibody (1:50; D18, Developmental Studies Hybridoma Bank, Iowa City, IA) pipetted onto individual sections. Sections

were rinsed in 0.01M PBS and incubated in TRITC fluorescent goat anti-mouse IgG (1:200; T-5393, Millipore Sigma, Milwaukee, WI). Slides were coverslipped with Vectashield Hardset mounting medium for Fluorescence (Vector), allowed to dry, the edges sealed with nail polish, and cured overnight at 4ºC. Slides were then stored in opaque slide boxes until examination.

AR quantification and analysis

Immunolabeled androgen receptor puncta were identifiable under FITC HYQ filtered fluorescent illumination (excitation 460-500µm; dichromatic mirror 505µm; barrier 510-560µm), while basal lamina membranes were identifiable under Texas Red HYQ filtered fluorescent illumination (Excitation 532-587µm; dichromatic mirror 595µm; barrier 608-683µm; Figs. 2.4-2.6) at x720 final magnification. To ensure that visualization under each fluorescent wavelength captured the same field of view, the image was first visualized under Texas Red HYQ to ensure that the field of view contained muscle fibers (identified by the presence of basal lamina staining). The fluorescent filters were then rotated while the microscope stage remained unmoved, allowing for FITC HYQ excitation through the same field of view to visualize androgen receptor-positive puncta. Images of basal lamina and androgen receptor puncta staining for each field of view were digitally captured separately in Stereo Investigator. The microscope stage was then moved to view the eight adjacent fields of view surrounding this initial field of view, and images were captured as described above. This process of capturing images for nine fields of view per section was repeated for a single representative section on each slide. Due to the sparse nature of androgen receptor expression in somatic skeletal muscle in normal animals (Monks et al., 2004), the first pseudorandomly selected field of view was chosen by the presence of at least one labeled androgen receptor.

Due to the transient nature of fluorescent signal, images for each field of view were digitally captured in Stereo Investigator under wavelengths illuminating each of androgen receptor-positive

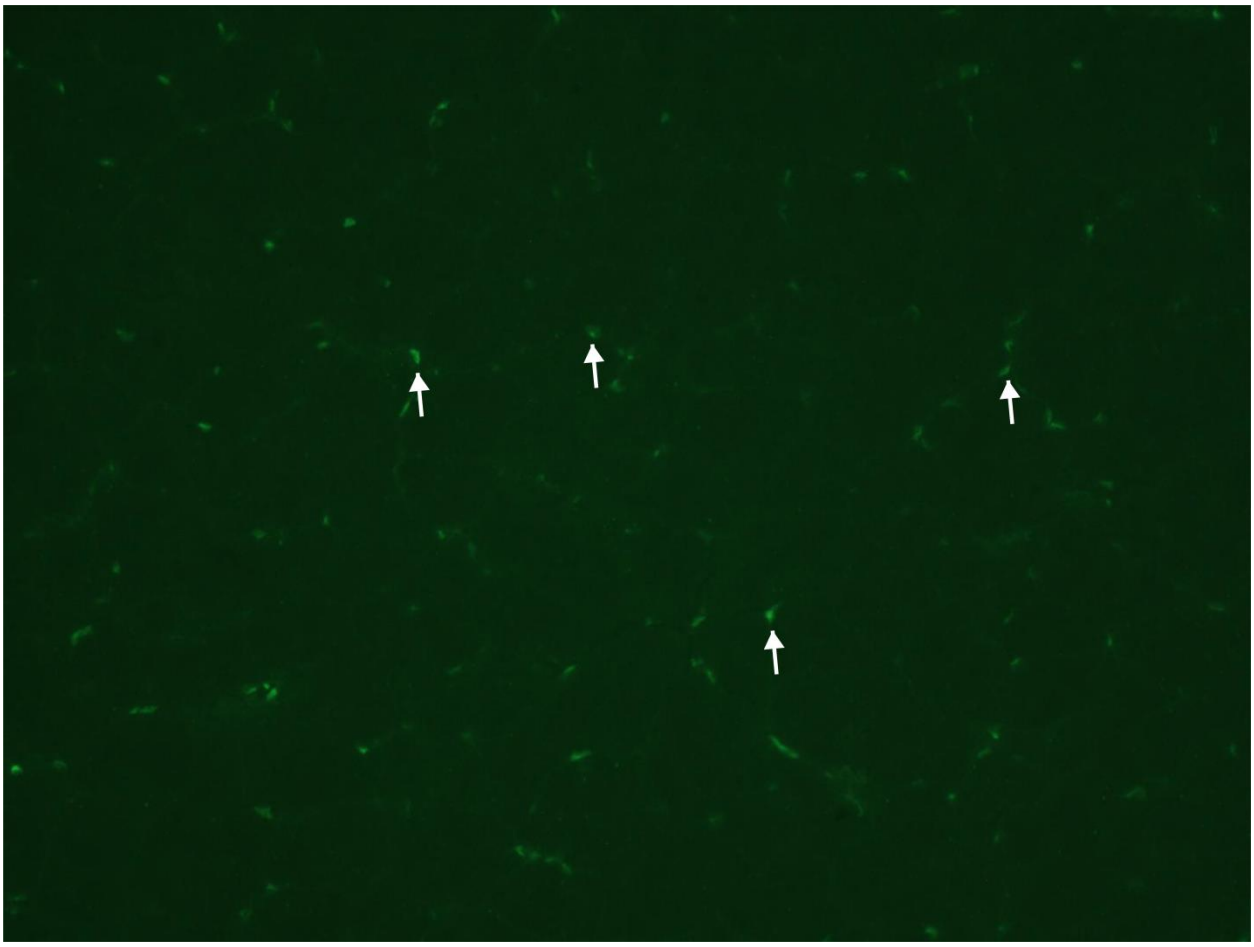

Figure 2.4. Captured image of fluorescently immunolabeled androgen receptor puncta in bulbocavernossu/levator ani muscle (BC/LA) tissue. BC/LA tissue was used the development of this immunohistochemical protocol, and was included here to illustrate the protocol's effiacy. Immunolabeled androgen receptor puncta arppear as bright green against the darker green background, and are indicated by white arrows.

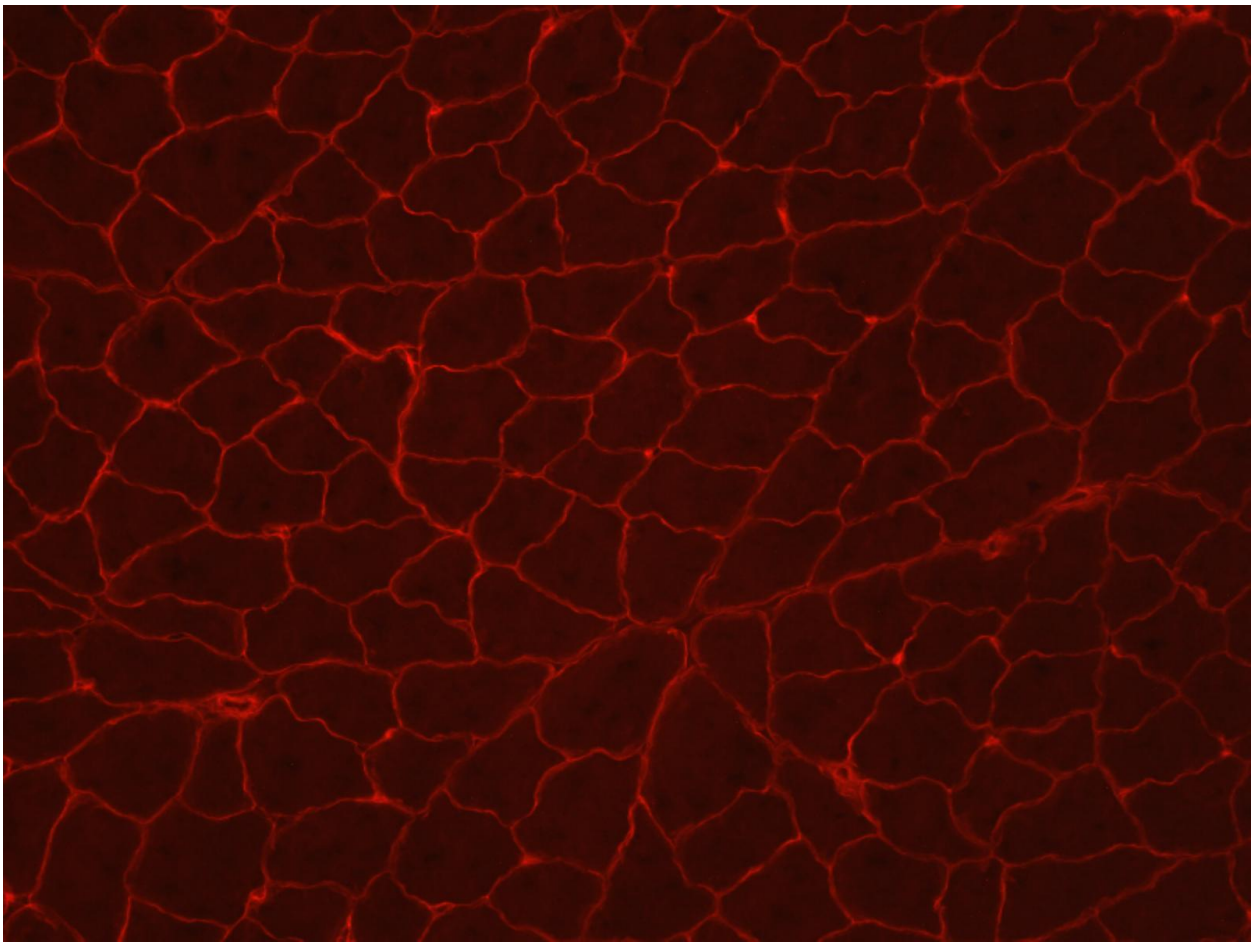

Figure 2.5. Captured image of fluorescently iimmunolabeled basal laminar membrane in bulbocavernossu/levator ani muscle tissue. Note that this image contains the exact same field of view as Fig. 2.4.

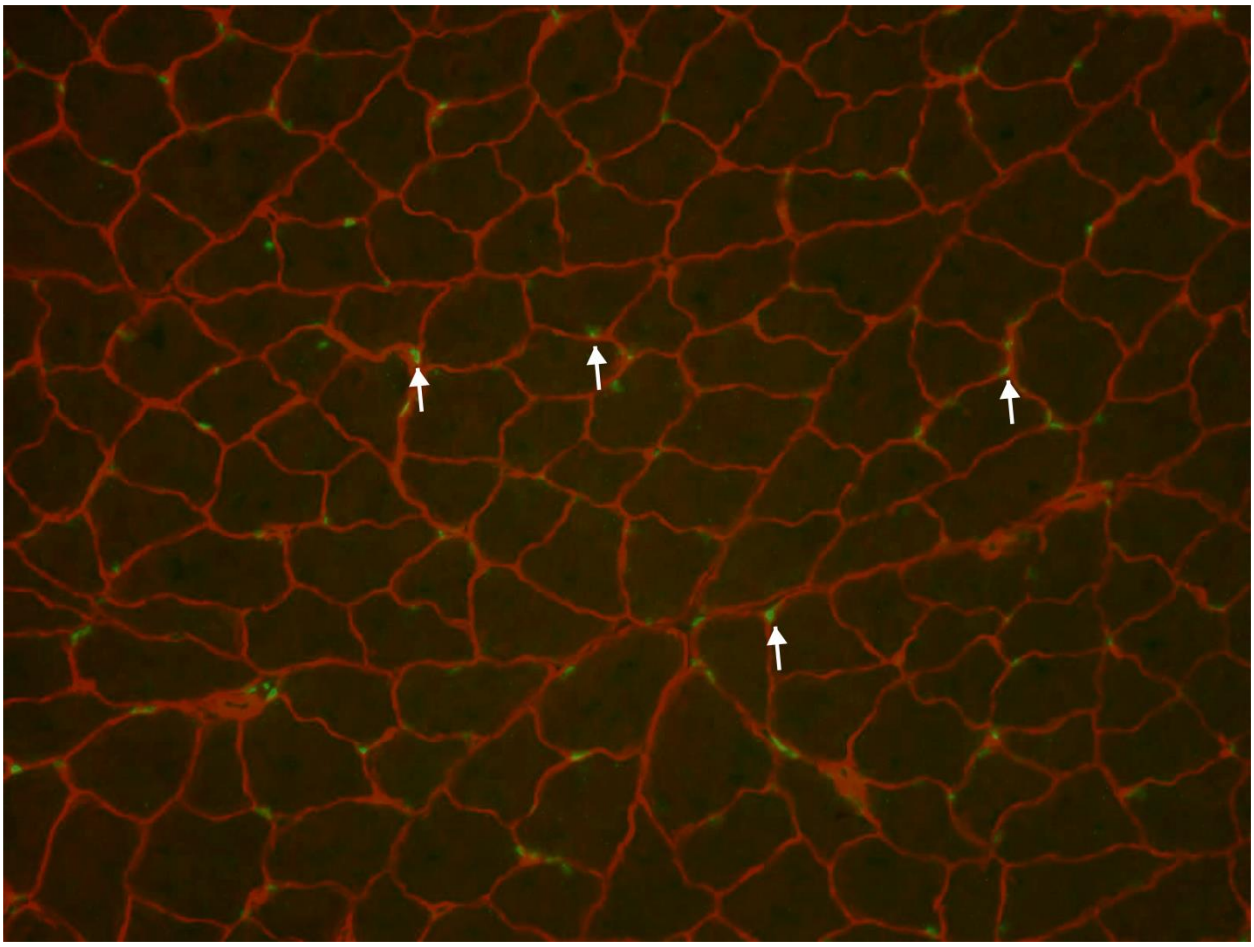

Figure 2.6. Merged image of bulbocavernosus/levator ani muscle tissue fluorescently immunolabeled for both basal laminar membrane (red) and androgen receptor puncta (green). This image is a merge of Figures 2.4 and 2.5, with the opacity of the superimposed basal laminar-labeled image (Fig .2.5) decreased to ~30% of original opacity to allow the green androgen receptor-positive puncta (Fig. 2.4) to show through. Androgen receptor-positive puncta are indicated by white arrows; arrows are pointing to the same androgen receptor-positive puncta indicated in Fig. 2.4.

puncta and basal lamina. These images were merged in Photoshop 5.5 (Adobe, San Jose, CA) by opening both images, superimposing the images with basal laminar label over that with androgen receptor-positive puncta, and adjusting the opacity of the basal laminar image so that androgen receptor-positive puncta were still visible while being able to identify the muscle fibers ringed by the basal lamina. Basal laminar image opacity was adjusted to 30-40% of original opacity.

Merged images were reimported to Stereo Investigator where the number of whole muscle fibers and androgen receptor-positive puncta were counted and area of muscle fibers calculated. The total number of androgen receptor-positive puncta from nine fields of view from a single animal were summed, and a measure of the density of androgen receptor-positive puncta was calculated by dividing the total number of androgen receptor-positive puncta per animal by the area of the field of view used to collect all images (each field measured 227852.46μm^2; total area sampled = 4101344.28μm^2), and converting the value to androgen receptor-positive puncta per square millimeter.

Muscle fiber areas were calculated using Stereo Investigator's Nucleator probe (Gundersen, 1988). A set of four rays emanating from a point randomly chosen within each muscle fiber was drawn and oriented randomly, and the point at which each ray intersected the basal laminar membrane was marked in Stereo Investigator. Cross-sectional areas were measured at a final magnification of 720x were then averaged for each animal for statistical analysis.

CHAPTER 3

IS EXERCISE NEUROPROTECTIVE AGAINST DENDRITIC ATROPHY OF SURVIVING QUADRICEPS MOTONEURONS FOLLOWING THE DEATH OF THEIR NEIGHBORS?

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Neurodegenerative diseases and neural injuries can result in the loss of spinal motoneurons, potentially leading to decreased quality of life or more severe pathologies. In addition to the consequences directly related to motoneuron loss, injured motoneurons that survive also show a variety of morphological and functional changes after injury. Previous work has examined the effects of motoneuron death on the structure and function of adjacent surviving motoneurons using a rat model of motoneuron death. These studies have demonstrated that surviving motoneurons respond to the loss of their neighbors with marked dendritic atrophy (Little et al., 2009; Cai et al., 2017; Chew et al., 2019), and this phenomenon occurs in both androgen-sensitive and more conventional somatic neuromuscular populations (Fargo and Sengelaub, 2004a,b; Little et al., 2009).

This induced atrophy is responsible for at least some of the movement deficits that accompany degenerative movement disorders and spinal cord trauma, as it results in reduced excitability of the remaining motoneurons (Little et al., 2009). Furthermore, it is likely that other adjacent motoneuron populations experience dendritic atrophy in addition to the observed atrophy in motoneurons innervating the vastus lateralis (VL), indicating that negative consequences of neural injuries or disease can have

rippling effects beyond primary symptoms. Given that we currently lack the technology to replace dead motoneurons, developing the ability to protect and promote recovery in surviving motoneurons from injury-induced atrophy is an important goal.

Treatment with gonadal steroid hormones promotes a wide array of neuroprotective and neurotherapeutic effects (Foecking et al., 2015). In our model of induced motoneuron loss, treatment with a physiological dose of exogenous testosterone attenuates dendritic atrophy, as well as the attenuated excitability, in motoneurons (Little et al., 2009; Chew et al., 2019). This effect of androgens is mediated via classical gonadal steroid receptor activation, and blockade of androgen receptors with flutamide completely prevents the neuroprotective effects from induced dendritic atrophy (Cai et al., 2017).

Exercise has also been demonstrated to be neurotherapeutic in multiple models of neuronal injury. These therapeutic effects include indirect effects, such as reduction of neuroinflammation following spinal cord injury (Sandrow-Feinberg and Houlé, 2015), and also more direct protective effects on the preservation of motoneuron dendritic structure (Gazula et al. 2004). In addition, exercise can also induce upregulation of neurotrophic factors that promote neuroplasticity [e.g., BDNF and glial-derived neurotrophic factor (GDNF); Vaynman and Gomez-Pinilla, 2005; McCullough et al., 2013; Gyorkos et al., 2014]. Exercise also increases both the number of regenerating axons sprouting from the proximal stump of axotomized fibular nerves and axon elongation in the injured peripheral nerve when compared to non-exercised animals (Sabatier et al., 2008).

Androgens have been directly implicated in the positive effects of exercise after injury. For example, treadmill training results in both increases in serum testosterone levels and enhancement of axon regeneration after axotomy in male rats; castration prevents treadmill training effects on axon regeneration (Wood et al., 2012). In the same axotomy model, systemic treatment of exercised animals with the androgen receptor antagonist flutamide reduces the median length of regenerating axons to

lengths comparable to those of animals who did not exercise (Thompson et al., 2014), suggesting that androgen receptor action is a necessary driver of the neuroprotective benefits of exercise following injury.

This androgen-dependency of exercise's neurotherapeutic effects following axotomy and the previously established mechanism that exogenous testosterone treatment relies on androgen receptor activation leads us to believe that exercise may be a viable substitute for exogenous androgen therapy following partial motoneuron loss. Thus, in this chapter of the thesis, we tested whether exercise was neuroprotective against induced dendritic atrophy following partial motoneuron depletion.

METHODS AND DESIGN

Animals

Adult male Sprague Dawley rats approximately 100 days old were used for this experiment. Saporin injections were made into the left vastus medialis (VM) muscle of the quadriceps as described in Chapter 2. Briefly, rats were anesthetized with isoflurane, and the left VM was exposed and injected with CTB-saporin (2 µL, 0.2%; Advanced Targeting Systems).

Some rats were not treated further (n = 6), whereas others were allowed *ad libitum* access to exercise wheels (width = 4.375 in; diameter = 14.5 in; circumference = 45.5 in) attached to their home cages (n = 11) immediately following saporin injections. Wheel revolutions were tracked daily to ensure that rats were engaging in exercise for the duration of the recovery period. To control for potential effects of exercise alone, a group of intact animals (n = 4) with access to exercise wheels was included, as well as a group of untreated control animals who received no exercise (n = 5). Because some of the animals in the study (overall n = 26) were not included in all analyses due to histological or histochemical compromise, group sizes for each analysis are reported individually below.

Tissue Processing and Histochemistry

Four weeks following saporin injection and beginning of exercise, rats were re-anaesthetized, the left VL exposed and injected with BHRP (2 µL. 0.2%, Invitrogen, Temecula, CA), as described in Chapter 2. Animals were sacrificed two days later, exsanguinated and fixed (1% paraformaldehyde/1.25% glutaraldehyde). To confirm the specificity of the saporin injections, the VM and VL were dissected bilaterally and weighed, and the lumbar spinal cords were removed and prepared for histochemical processing. Spinal cords were post-fixed and cyroprotected overnight, then embedded in gelatin, frozen, and sectioned transversely at 40µm into four alternate series. One series was stained with thionin for use in cell counts, and the remaining three series were immediately reacted to visualize BHRP using the tetramethyl benzidine protocol described in Chapter 2. Once reacted, sections were mounted on gelatin-coated slides, and counterstained with thionin.

Microscopy

Motoneuron counts

Motoneuron counts were collected in order to confirm that CTB-saporin injection was effective in inducing motoneuron death. The method is more extensively detailed in Chapter 2. In summary, thionin-stained motoneurons in the left and right lateral motor columns were stereologically counted along the rostrocaudal distribution of BHRP-labeled motoneurons. Raw counts were corrected for sampling, and a ratio of the number of motoneurons in the left and right lateral motor columns was calculated in order to determine whether there were fewer motoneurons on the CTB-saporin injected left side when compared to the uninjected right side. This has proven to be a reliable indicator of assessing whether CTB-saporin successfully induced the death of motoneurons (Little et al., 2009; Cai et al., 2017; Chew et al., 2019).

Motoneuron counts were derived from a mean of 11.08 sections spaced 480 µm apart and distributed uniformly through the entire rostrocaudal extent of the quadriceps motoneuron pool range.

This sampling scheme produced an average estimated coefficient of error (CE) of .053. (untreated, n = 5; SAP, n = 6; SAP+EXERCISE, n = 10; intact+EXERCISE, n = 4).

Using similar methods, the number of BHRP-filled motoneurons was assessed in all sections of the reacted series through the entire rostrocaudal extent of their distribution for all animals. Counts of BHRP-labeled quadriceps motoneurons were made under brightfield illumination, where somata could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed (untreated, n = 5; SAP, n = 6; SAP+EXERCISE, n = 6; intact+EXERCISE, n = 4).

Motoneuron Morphometry

Measures of motoneuron morphometry were collected from one of the three series that underwent histochemical processing to visualize BHRP label. Motoneurons labeled with BHRP were counted in a similar fashion described above for cell counts in the non-histochemically reacted series. Measures of dendritic morphometry were collected by reconstructing BHRP-labeled dendritic arbors from individual sections and merging all reconstructed sections from a single animal into a single composite reconstruction. This composite was used to find the summed total length of labeled dendrites and analyze the radial distribution, radial extent, and rostrocaudal extent of dendritic label. These methods and their rationale are described in detail in Chapter 2 (untreated, n = 5; SAP, n = 6; SAP+EXERCISE, n = 6; intact+EXERCISE, n = 4).

RESULTS

Running Performance

Animals ran consistently over the four weeks they were allowed access to running wheels, averaging 3920 ± 366 (mean ± SEM) revolutions per day (4.51 ± 0.42 kilometers; Fig. 3.1). Injection of saporin had no effect on exercise, and average daily running wheel revolutions did not differ from those

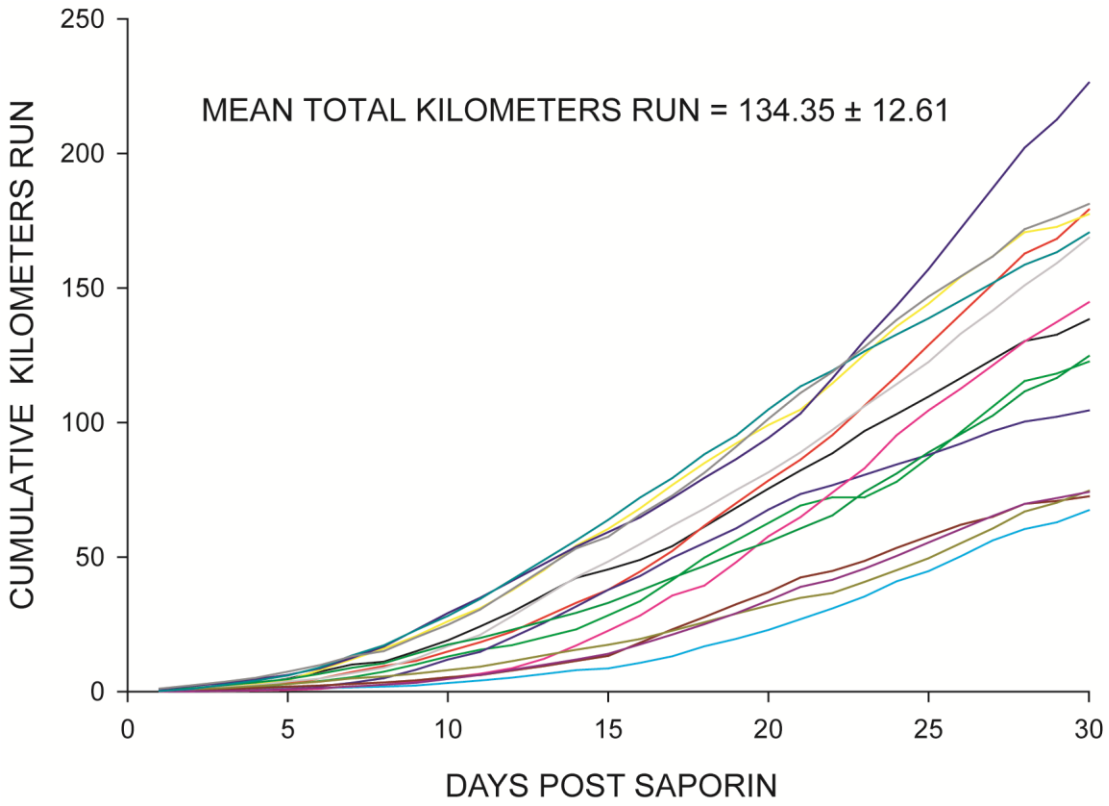

Figure 3.1. Total cumulative distance run in the 30 days following injection of saporin; each line represents the cumulative kilometers run by a single rat. Rats showed a fair amount of variation in both the total cumulative distance run and in the progressive increase in daily distance run. However, there were no group differences in either total cumulative distance or daily distance run.

of intact animals [$F(1,13) = 1.22$, *ns*]. Overall, animals ran an average cumulative total of 134.35 ± 12.61 kilometers over the four weeks of *ad lib* exercise.

Muscle Weights

Differences in body weight were present across groups [$F(3,22) = 12.15$, $p < 0.0001$], and thus raw muscle weights were corrected for body mass to assess potential effects of saporin and/or exercise on muscle weight (Fig. 3.2). In untreated animals, the corrected weights of the right (0.17 ± 0.01) and left (0.17 ± 0.01) VM muscles were similar [$t(4) = 2.14$, *ns*]. Although the weights of the uninjected (right) VM muscles were not affected [$F(3,22) = 2.73$, *ns*], injection of saporin into the left VM resulted in notable muscle atrophy across the saporin groups [overall average of 68% reduction in weight; $F(3,22) = 69.34$, $p < 0.0001$]. Compared to those of untreated animals, saporin-injected animals had VM weights that were 74% lighter (LSD, $p < 0.0001$). Exercise did not prevent muscle weight loss; compared to those of untreated animals, saporin-injected rats allowed to exercise has VM weights that were 62% lighter (LSD, $p < 0.0001$). Muscle weights across saporin groups did not differ from each other (LSD, *ns*).

The effect of saporin injection on quadriceps weight was specific to the injected muscle. In untreated animals, the corrected weights of the right (0.40 ± .02) and left (0.41 ± .02) VL muscles were similar [paired t-test, $t(4) = .43$, *ns*]. The weights of the VL muscles on the untreated (right) side did not differ across groups [$F(3,22) = .74$, *ns*]. Most importantly, the weights of the VL muscles adjacent of the saporin-injected VM muscles also did not differ across groups [$F(3,22) = .41$, *ns*]. Exercise had no effect on VL muscle weight (LSDs, *ns*).

Motoneuron Counts

In untreated animals, the number of motoneurons within the identified quadriceps range did not differ between the left (251.2 ± 14.28) and right (237.6 ± 24.97) motor column [paired t-test, $t(4) = 0.63$, *ns*]. Motoneuron counts indicated that saporin was effective in inducing partial motoneuron depletion

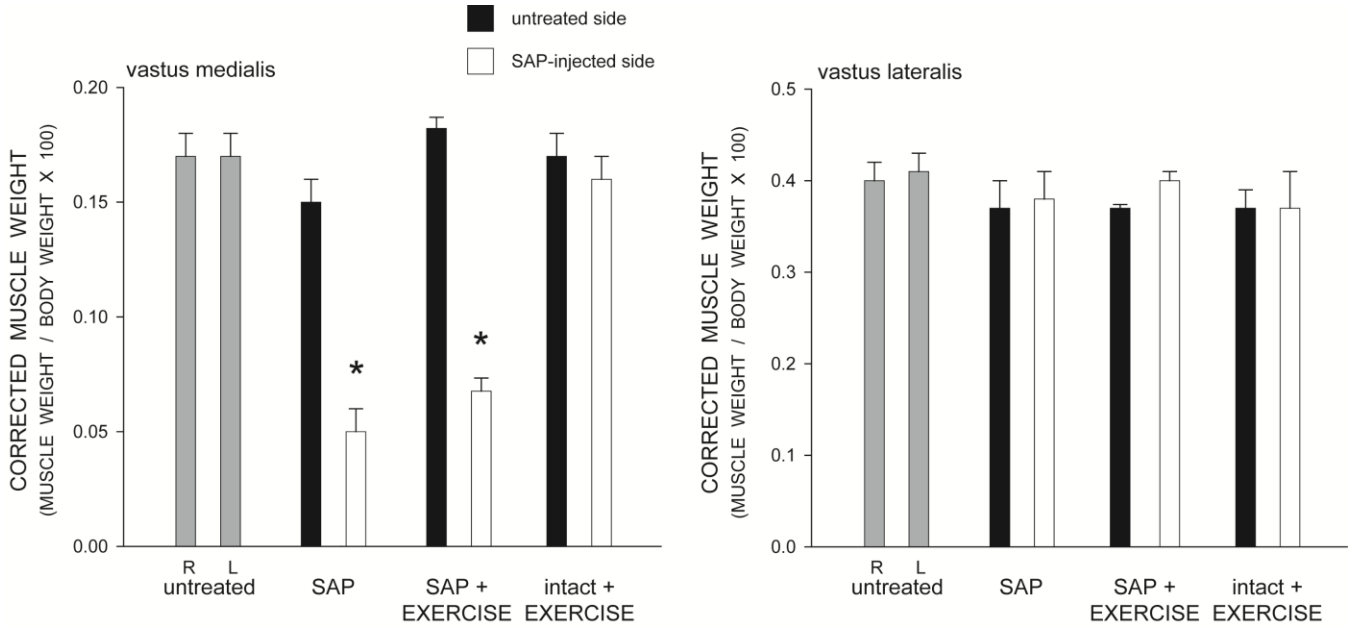

Figure 3.2. Weights of the vastus medialis muscles corrected by body weight in untreated animals, saporin-injected animals that either received no further treatment (SAP) or were given *ad lib* exercise (SAP+EXERCISE), and intact animals given *ad lib* exercise (intact+EXERCISE) at four weeks after saporin injection. Gray bars represent weights from the right (R) and left (L) sides in untreated animals. Black bars represent weights from the untreated contralateral (right) side of the quadriceps muscle, and white bars represent weights from the saporin injected (left) side of the quadriceps muscle of saporin-injected animals. Saporin injection reduced the weight of the vastus medialis muscle; exercise had no effect on muscle weight. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$).

from the quadriceps motor pool (Fig. 3.3). Injection of saporin into the left VM muscle resulted in the death of ipsilateral quadriceps motoneurons, significantly reducing the number of motoneurons in the left motor column relative to that of the right [$F(3,21) = 4.59$, $p < 0.02$]. Unilateral injection of saporin into the left VM resulted in a 21% reduction in the relative number of motoneurons compared with that of untreated animals (LSD, $p < 0.009$). Exercise did not prevent this reduction (overall average of 21% reduced; LSDs, $p < .009$ compared to untreated animals). As expected, exercise had no effect on motoneuron number in intact animals (LSD, *ns*).

Motoneuron Morphometry

Injection of BHRP into the left VL successfully labeled ipsilateral quadriceps motoneurons in all groups (Fig. 3.4). The dendritic arbor of quadriceps motoneurons was strictly unilateral, with extensive ramification along the ventrolateral margins of the gray matter and in the lateral funiculus, as well as throughout the ventral horn. An average of 36.95 (± 3.83) motoneurons per animal was labeled with BHRP, and this did not vary across groups [$F(3,17) = 1.03$, *ns*].

Dendritic Length

After saporin-induced motoneuron death, surviving neighboring quadriceps motoneurons underwent marked dendritic atrophy (Fig. 3.5). Dendritic length was decreased by 64% in saporin-injected animals compared to that of untreated animals [LSD, $p < .0001$; overall test for the effect of group on dendritic length $F(3,17) = 9.67$, $p < .0006$]. However, whereas dendritic length in saporin-injected animals who exercised were also shorter than that of untreated animals (LSD, $p < 0.04$), exercise attenuated dendritic atrophy, with dendritic length being reduced on average by only 28%. Compared with saporin animals who received no further treatment, saporin-injected animals who exercised had dendritic lengths were 97% longer (LSD, $p < 0.02$). Exercise in intact animals had no effect on dendritic length (LSD, *ns*).

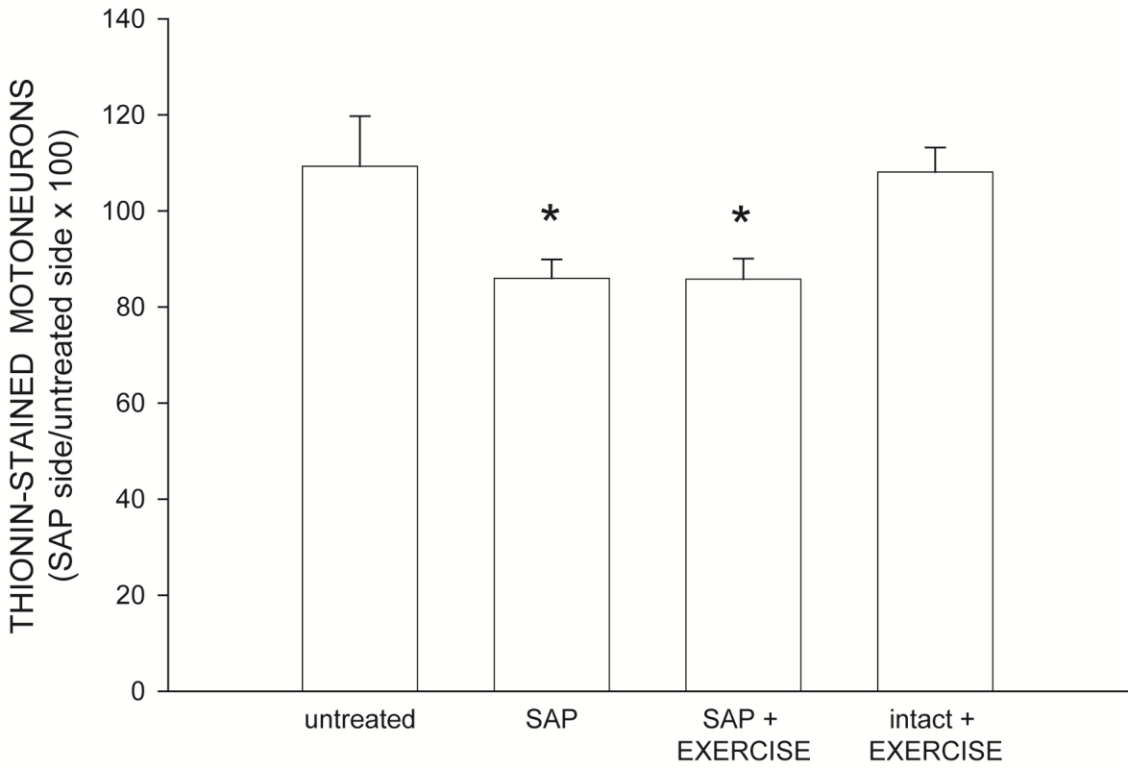

Figure 3.3. Numbers of quadriceps motoneurons in untreated animals, saporin-injected animals that either received no further treatment (SAP) or were given *ad lib* exercise (SAP+EXERCISE), and intact animals given *ad lib* exercise (intact+EXERCISE) at four weeks after saporin injection, expressed as a ratio of motoneuron number ipsilateral to the saporin-injected muscle relative to that on the untreated side. Saporin killed approximately 21% of the ipsilateral quadriceps motoneurons, regardless of subsequent treatment. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$).

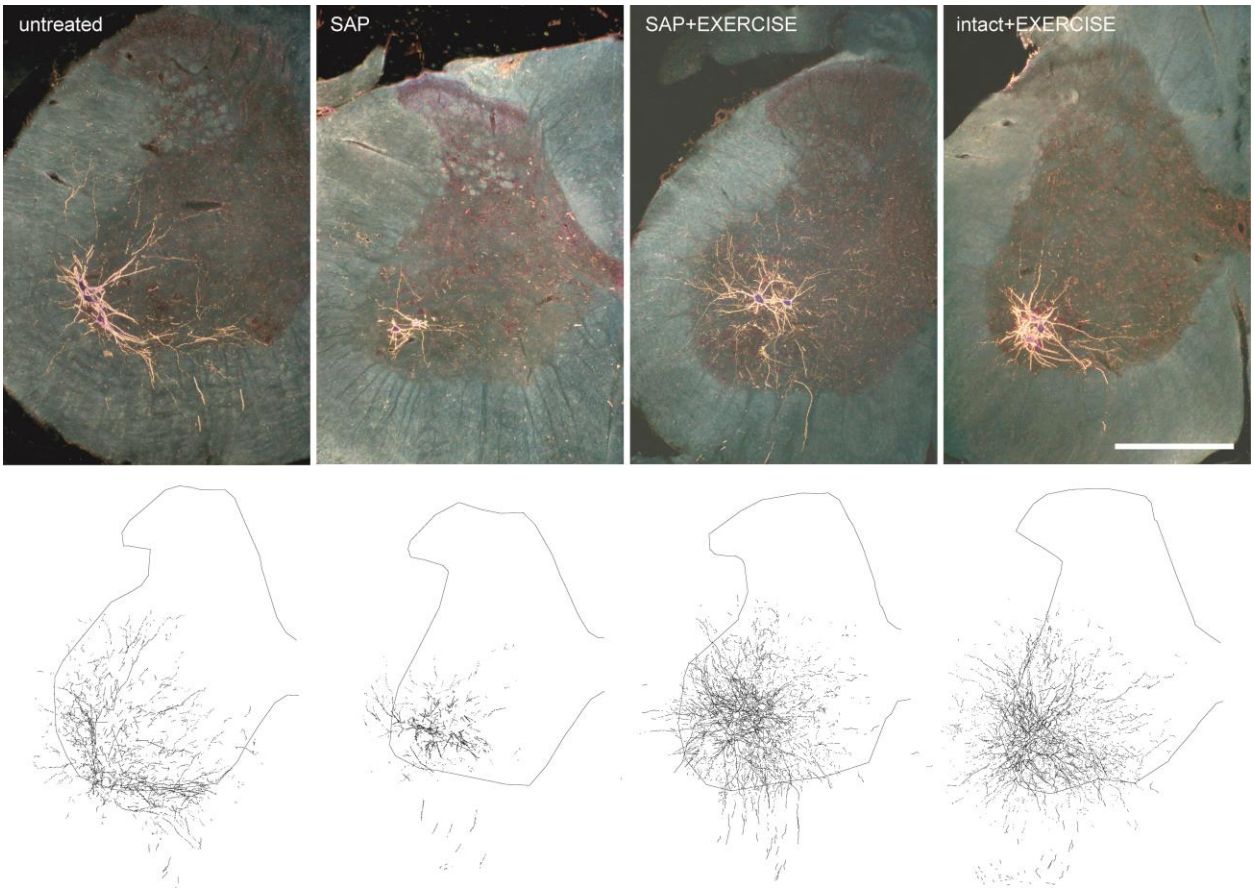

Figure 3.4. Darkfield digital micrographs of transverse hemisections through the lumbar spinal cords and computer-generated reconstructions BHRP-labeled somata and processes of an untreated animal, and saporin-injected animals with either no further treatment (SAP) or given *ad lib* exercise (SAP+EXERCISE), and an intact animal given *ad lib* exercise (intact+EXERCISE) after BHRP injection into the left vastus lateralis muscle. Computer-generated composites of BHRP labeling were drawn at 480 μm intervals through the entire rostrocaudal extent of the quadriceps motor pool; these composites were selected because they are representative of their respective group average dendritic lengths. Scale bar = 500 μm.

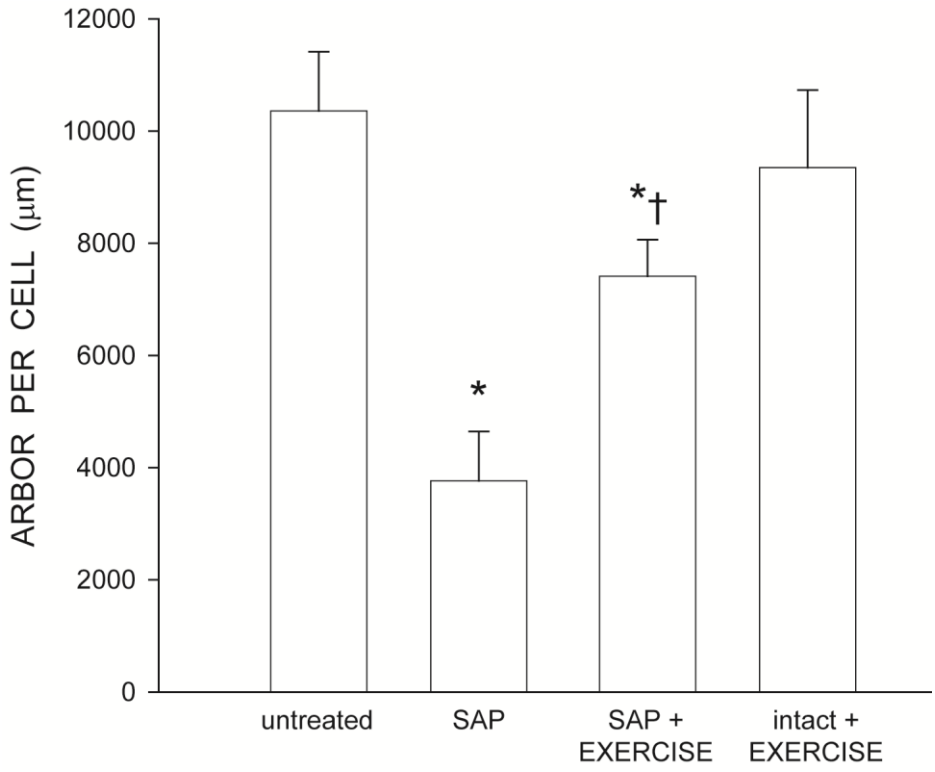

Figure 3.5. Dendritic lengths of quadriceps motoneurons in untreated animals, saporin-injected animals that either received no further treatment (SAP) or were given *ad lib* exercise (SAP+EXERCISE), and intact animals given *ad lib* exercise (intact+EXERCISE). Following saporin-induced motoneuron death, surviving neighboring motoneurons lost almost 64% of their dendritic length. Exercise attenuated this dendritic atrophy, but had no effect in intact animals. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$). † indicates significantly different from untreated saporin-injected animals ($p < 0.05$).

Dendritic Distribution

Dendritic length was non-uniformly distributed across radial bins, and a repeated measures ANOVA revealed a significant effect of radial location [$F(11,187) = 13.46$, $p < 0.0001$; Fig. 3.6]. Consistent with the results seen in total dendritic length analysis, there was also a significant effect of group [$F(3,187) = 10.52$, $p < 0.0005$]. There were reductions in dendritic length throughout the radial distribution, ranging from 38% (180º to 240º) to 79% (60º to 120º) in saporin-injected animals compared with untreated animals [$F(1,99) = 26.19$, $p < .0007$]. Saporin-injected animals allowed to exercise showed an attenuation of these reductions, with reductions in dendritic length ranging from no change (180º-300º) to 54% (60º to 120º) compared to untreated animals [$F(1,99) = 9.44$, $p < .02$]. Dendritic lengths per bin in exercised saporin-injected animals were longer than those of saporin-injected animals who received no further treatment [$F(1,110) = 9.70$, $p < 0.02$] throughout most of the radial distribution, with increases ranging from 61% (180º-240º) to 120% (240º-300º).

Dendritic Extent

In agreement with the nonuniform dendritic distribution of quadriceps motoneurons apparent in Fig. 3.6, radial extent differed across bins (Fig. 3.7), and a repeated measures ANOVA revealed a significant effect of location [$F(11,187) = 14.81$, $p < 0.0001$]. However, radial dendritic extent did not differ across groups [$F(3,187) = 2.10$, *ns*].

Rostrocaudal dendritic extent also did not differ across groups [$F(3,17) = 2.30$, *ns*], spanning 3776.0 ± 546.3 µm in untreated animals, 3760.0 ± 402.1 µm in saporin-injected animals who received no further treatment, 2760.0 ± 336.0 µm in exercised saporin-injected animals, and 2700.0 ± 326.8 in exercised intact animals.

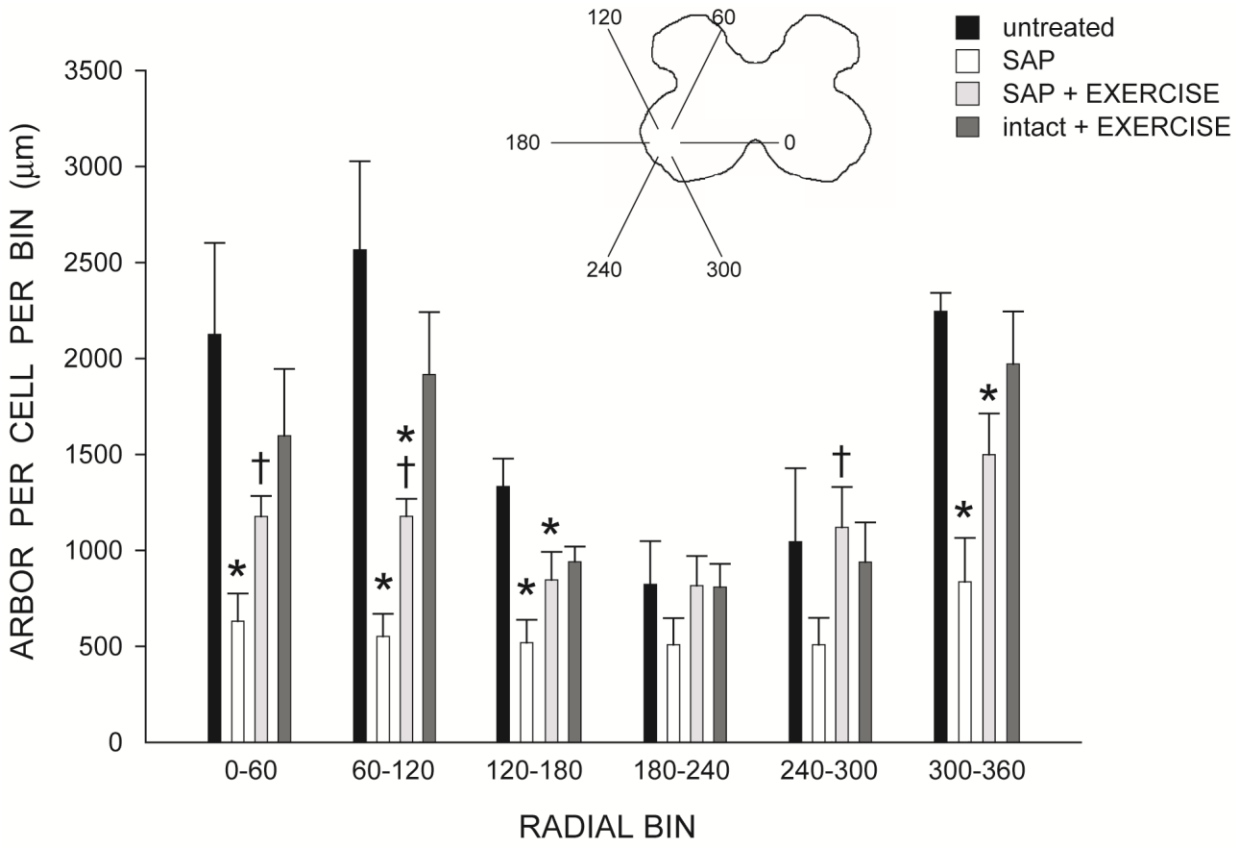

Figure 3.6. Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron dendritic distribution. Length per radial bin of quadriceps dendrites in untreated animals (black bars), saporin-injected animals that either received no further treatment (SAP, white bars) or were given *ad lib* exercise (SAP+EXERCISE, light gray bars), and intact animals given *ad lib* exercise (intact+EXERCISE, dark gray bars). For graphic purposes, dendritic length measures have been collapsed into 6 bins of 60° each. Quadriceps motoneuron dendritic arbors display a non-uniform distribution, with the majority of the arbor located between 300° and 120°. Following saporin-induced motoneuron death, surviving neighboring motoneurons had reduced dendritic length throughout the radial distribution. Exercise attenuated this reduction, but had no effect in intact animals. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$). † indicates significantly different from untreated saporin-injected animals ($p < 0.05$).

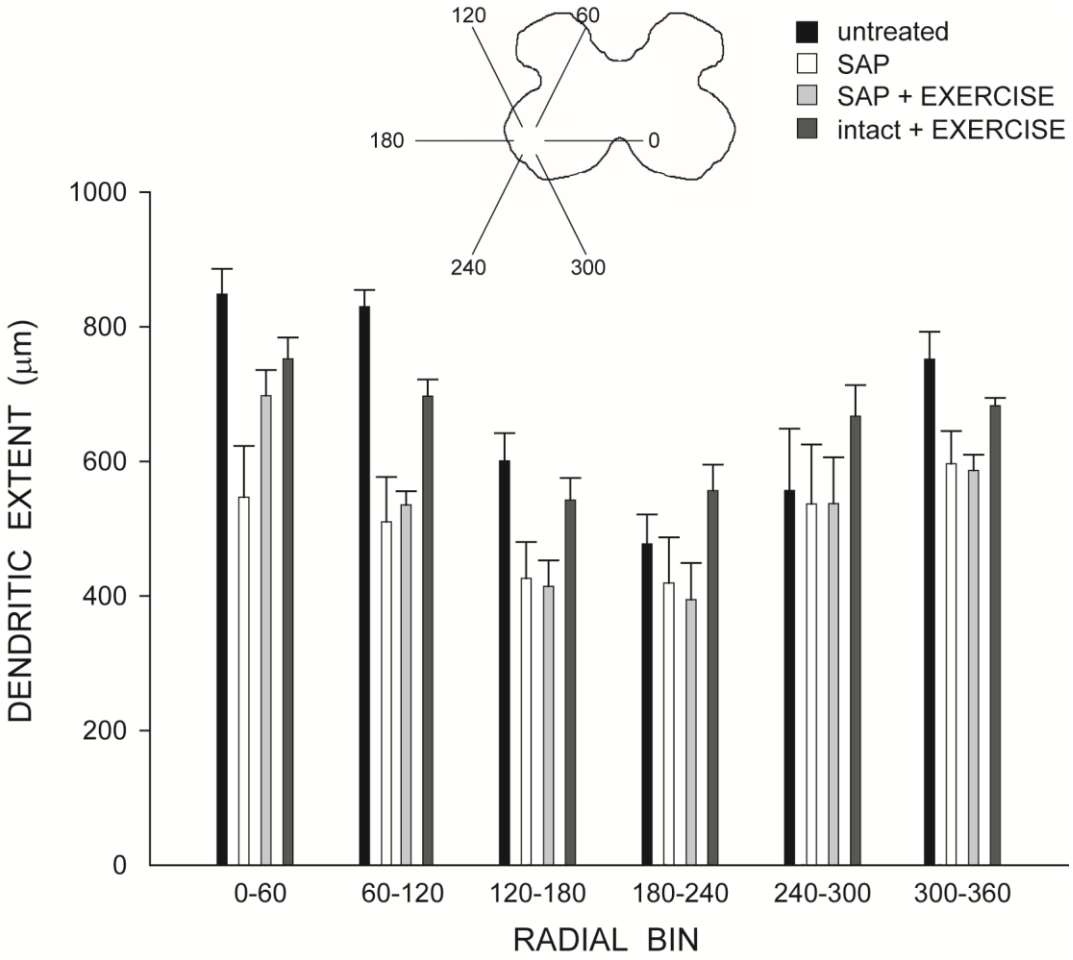

Figure 3.7. Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron radial dendritic extent. Radial extents of quadriceps dendrites in untreated animals (black bars), saporin-injected animals that either received no further treatment (SAP, white bars) or were given *ad lib* exercise (SAP+EXERCISE, light gray bars), and intact animals given *ad lib* exercise (intact+EXERCISE, dark gray bars). For graphic purposes, dendritic extent measures have been collapsed into 6 bins of 60° each. Extent measures did not differ across groups. Bar heights represent means ± SEM.

DISCUSSION

Surviving motoneurons respond to the death of neighboring motoneurons with marked dendritic atrophy (Little et al., 2009). Treatment with testosterone, mediated by classical receptor activation, is protective against this atrophy (Cai et al., 2017). In this study, I demonstrate that exercise attenuates induced motoneuron dendritic atrophy to a degree similar to that seen with supplemental testosterone treatment.

Saporin Injection and Muscle Weights

Saporin injection into the VM significantly decreased muscle weight (68%) in a manner that was consistent with previous findings (Little et al., 2009; Cai et al., 2017; Chew et al., 2019). Intramuscular injection of saporin had no effect on the adjacent, uninjected VL muscle. Thus, saporin injections were effective at the targeted VM and did not spread to the adjacent VL, an important consideration for interpreting the effects seen on the morphology of *surviving* motoneurons. Furthermore, there were no differences in the number of BHRP labeled motoneurons following injection of BHRP into the VL, indicating that loss of motoneurons innervating the VM did not affect the ability to visualize VL motoneurons.

Exercise had no effect on preventing saporin-induced decreases in weight of the injected VM muscle, nor did it prevent saporin-induced motoneuron death. This is consistent with previous findings concerning treatment with testosterone (Fargo and Sengelaub, 2004a,b; Little et al., 2009; Chew et al., 2019). Thus, the beneficial effects of exercise on the morphology of neighboring surviving motoneurons cannot be attributed to differences resulting from the ability of saporin to kill motoneurons.

Although saporin injection was effective at both killing innervating motoneurons and reducing target muscle mass, it did not impair the ability of the saporin-treated rats to exercise. Running wheel performance in saporin-treated animals did not differ from that of intact animals, and animals in both groups ran an impressive overall cumulative distance of almost 135 kilometers over the four weeks of

treatment. Thus, the exercise manipulation was successful, and provided a neurotherapeutic treatment to the saporin-injected rats.

The lack of difference in distance run between intact exercised and saporin-injected exercised animals does raise some interesting questions regarding the role of individual muscles of the quadriceps and how they contribute to running behavior. It is possible that this phenomenon is due to the relative contributions of the individual muscles of the quadriceps. For example, the VM is quite small compared to the VL and rectus femoris muscles that also make up the quadriceps complex, and these larger muscles presumably produce more contractile force as the quadriceps activates. Thus, it is possible that had saporin been injected to the VL, and the resulting loss of a larger muscle producing more force were to occur, running behavior would be more severely impacted by our saporin manipulation.

Protection from Dendritic Atrophy

Consistent with our previous studies, saporin-induced motoneuron death resulted in a pronounced dendritic atrophy in surviving nearby quadriceps motoneurons (Little et al., 2009; Cai et al., 2017; Chew et al., 2019). Previous experiments using exogenous testosterone treatment have ruled out that this dendritic atrophy is the result of the loss of afferent fibers from the saporin-injected muscle (Cai et al., 2017), or the pronounced increase in activated microglia in the quadriceps motor pool following saporin-induced motoneuron death (Chew et al., 2019). My lab's prior publications have speculated that the induced death of motoneurons could result in the release of toxins [e.g., inflammatory cytokines (IL-6, IL-1β, TNF-α), purines (ATP), glutamate, and matrix metalloproteinases (MMPs)] into the extracellular space near the dendrites of the surviving motoneurons, resulting in direct damage to the dendrites themselves (Fargo and Sengelaub, 2004a,b; Chew et al., 2019). Such local changes in the neuropil would be consistent with the general atrophy seen throughout the dendritic distribution, although whether any hypothetical toxins are released by the dying motoneurons, adjacent glia, or other

sources remains unknown. Alternatively, such toxins could harm the motoneuron more generally, resulting in dendritic retraction due to more holistic changes to the health of motoneurons.

These possible explanations as to the cause of the observed dendritic atrophy also provide possible explanations as to the mechanism of how exercise is conferring neuroprotective effects to the motoneurons. Exercise upregulates both antioxidant enzymatic activity (Powers et al., 1994) and the presence of heat shock proteins in skeletal muscle (Salo et al. 1991; Kregel, 2002). These upregulations are theorized to be adaptive mechanisms in response to the oxidative stress and other biochemical changes experienced during exercise, and thus could contribute to a neuroprotective effect on motoneuron dendrites.

Interestingly, many of these effects of exercise are also regulated by androgens. Testosterone regulates proteins thought to be involved in neuroprotection, including expression of heat shock proteins (Zhang et al., 2004; Tetzlaff et al., 2007) and proteins with antioxidant functions (e.g., catalase; Ahlbom et al., 2001). Furthermore, both testosterone (Jones and Oblinger, 1994; Verhovshek and Sengelaub, 2013) and exercise (Gomez-Pinilla et al., 2002; Vaynman and Gomez-Pinilla, 2005; Ding et al., 2006) have also been associated with upregulation of neurotrophic factors that promote neuroplasticity (e.g., BDNF) and the cytoskeletal protein β-tubulin in neurons.

The protection from induced dendritic atrophy in surviving quadriceps motoneurons obtained in the present study through *ad lib* exercise was remarkably similar to what we have previously reported through treatment with exogenous testosterone (Little et al., 2009; Cai et al., 2017; Chew et al., 2019). It is tempting to speculate that the similarity in protection from induced dendritic atrophy in surviving motoneurons seen with both exercise and androgen treatment indicates that these treatments are acting through a common mechanism. Such a common mechanism has been hypothesized for the effects of exercise and androgens on axonal regeneration following axotomy (an “in series mechanism”; Thompson et al., 2014). As described above, androgens have been directly implicated in the positive

effects of exercise after injury (Wood et al., 2012; English et al., 2014; Thompson et al., 2014), and exercise has commonly been associated with testosterone and its role in anabolic muscle growth (Bhasin et al. 2001a, 2003). Exercise training also results in elevations in serum testosterone (Kindermann et al., 1982; Spiering et al., 2009; Wood et al., 2012), although intensity, duration, prior conditioning of the subject, time point of measurement (e.g., immediately after exercise, hours after exercise, at rest, etc.; Tremblay et al., 2005; Vingren et al., 2010), or the type of exercise training (Tremblay et al., 2004; Sato and Iemitsu, 2015) all modulate serum testosterone concentrations.

Together, this collection of phenomena linking exercise, androgens, and resulting effects on neuromuscular systems suggests that it may be feasible to take principles derived from those individual variables and merge them into a unified theory of how behaviors, hormones, and other biological substrates work together to modulate neuromuscular structure, either throughout the normal course of life or following injury. For example, it has been established that exercise increases circulating concentrations of testosterone (Wood et al., 2012), spinal expression of BDNF (Gomez-Pinilla et al., 2001, 2002), and that androgens and BDNF interact at both the muscle and spinal motoneuron to regulate dendritic morphology (Verhovshek et al., 2010). Each of these observations carries independent value, but taken together they can paint a much more complete picture as to how hormone and neurotrophins are influenced by behavior to produce changes in neuronal structure. The 'natural' role of such as system could be imagined as such: exercise causes upregulation of serum testosterone, which allows for a larger number of androgen receptors to be activated at the muscle to induce exercise-driven adaptations to muscle fiber size or composition, which then necessitates changes to the innervating motoneuron structure driven by changes in muscular or neuronal BDNF expression. To remain on track with this thesis's theme of neuroprotection, one can substitute in 'attenuates atrophy of motoneuron dendrites' in for 'changes to the innervating motoneurons structure…' This calculus could be further complicated by the introduction of temporal effects of chronic vs. acute exercise programs, or

differences in testosterone signaling due to age or gender. If true, this would indicate that there are not separate mechanisms for neuroprotection via androgen treatment and neuroprotection via exercise, but rather that exercise is simply a behavioral means of inducing changes to the endocrine system that enact the adaptive physiological alterations induced by exercise.

Potential Mechanism of Action for Neuroprotection by Exercise

Such speculations concerning unified theories involving multiple interacting factors are interesting, but many concrete questions require answers prior to placing faith in such grand designs. It has been established in the present experiment that exercise is neuroprotective to motoneuron dendritic structure to a degree that is similar to that seen with testosterone treatment. While no studies have investigated the mechanism of how exercise is neuroprotective in this injury model, several studies have examined the mechanism of action regarding that of exogenous testosterone treatment. For instance, it has been found that exogenous testosterone treatment is dependent on classical steroid receptor activation (Cai et al., 2017) and that the necessary site of action of these receptors is at the target muscle of the observed motoneurons (Chung, 2015). There is also evidence that neurotherapeutic benefits of exercise are linked to androgen action; administration of the androgen receptor antagonist flutamide prevents the exercise-driven acceleration of axonal regeneration following axotomy (Thompson et al., 2014). While not conclusive, this does provide evidence that the neurologically beneficial effects of exercise can be linked to androgen action.

If my proposed theory of exercise and testosterone treatment sharing a common mechanism of neuroprotection is true, then this would indicate that exercise is also dependent on both the presence of androgens and receptor activation at the target muscle in order to protect motoneuron dendrites. These questions are addressed in Chapter 5 of this thesis.

CONCLUSIONS

The current experiment examined whether exercise is neuroprotective to surviving motoneurons following the induced death of their neighbors. Findings indicate that exercise is, in fact, neuroprotective to motoneurons following the death of their neighbors, reducing dendritic atrophy to a degree comparable to that seen with exogenous testosterone treatment. Previous work investigating the neuroprotective mechanism of exogenous testosterone treatment following induced motoneuron death has identified both a receptor dependency and a specific site of action where androgen receptor activation is necessary for testosterone to be neuroprotective. Thus, further research into the mechanism(s) of how exercise confers this neuroprotective effect to motoneurons may lead to the development and optimization of physical therapy regimens, or insight as to how exercise or other interventions may be effective following neural injury or disease.

CHAPTER 4

IS NEUROPROTECTION OF MOTONEURON DENDRITES BY EXERCISE DEPENDENT ON ANDROGEN ACTION?

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As mentioned in prior chapters of this dissertation, the loss of motoneurons has adverse effects on the structure and function of surviving motoneurons using a rat model of motoneuron death, including dendritic atrophy and a resulting decrease in electrophysiological excitability (Little et al., 2009; Cai et al., 2017). This induced atrophy and decrease in excitability is responsible for at least some of the movement deficits that accompany disease or injury-related loss of motoneurons, and makes protecting surviving motoneurons from injury-induced atrophy an important goal.

Treatment with gonadal steroids is neuroprotective in a variety of contexts (Foecking et al., 2015), and it has been specifically established that treatment with exogenous testosterone is protective against induced dendritic atrophy and reduced excitability following the death of adjacent motoneurons (Fargo and Sengelaub, 2004a,b; Little et al., 2009). This effect of androgens is mediated via classical receptor activation, and systemic blockade of androgen receptors completely prevents the neuroprotective effects (Cai et al., 2017). This suggests that receptor action is a necessary driver of the neuroprotective benefits of androgens.

In Chapter 3, I demonstrated that *ad lib* exercise is neuroprotective against dendritic atrophy, to a degree comparable to that seen when rats are treated with testosterone (Chew and Sengelaub, 2019). Many of the adaptive effects seen in muscle following exercise have been linked to androgen signaling pathways (Bhasin et al., 2001a, 2003; Aizawa et al., 2010; Hedayatpour and Falla, 2015). Administration of supraphysiological dosages of testosterone combined with exercise has been shown to increase lean muscle mass and muscle cross sectional area in human males (Bhasin et al., 1996, 2001a). Exercise with supplementary testosterone alters skeletal muscle synthesis and oxidation of glycogen compared to similarly exercised rats who did not receive testosterone (van Breda et al., 1993). Exercise accelerates axon sprouting and regeneration following axotomy via an androgen-dependent manner (Thompson et al., 2014). The similarity between the neuroprotective efficacy of exercise and exogenous testosterone treatment, the preexisting links between androgens and exercise adaptations, and the previously reported androgen dependency of exercise in other documented neurotherapeutic effects leads me to believe that the neuroprotective effects of exercise may be dependent on androgen action. Thus, this chapter will address whether the neuroprotective effects of exercise following partial motoneuron depletion depend on the presence of testes, presumably through their secretion of androgens.

METHODS AND DESIGN

Animals

Adult male Sprague-Dawley rats approximately 100 days old were used for this experiment. We used the toxin saporin, conjugated to the cholera toxin B subunit (CTB-saporin), to kill motoneurons, as described in Chapter 2. Briefly, rats were anesthetized with isoflurane, the left vastus medialis (VM)

muscle was exposed and injected with CTB-saporin (2 µL, 0.1%; Advanced Targeting Systems, Inc., San Diego, CA).

Some rats were not treated further (n = 6), whereas others were immediately allowed free access to exercise wheels (width = 11.2 cm; diameter = 37 cm; circumference = 116 cm) attached to their home cages (n = 11). To assess whether exercise is neuroprotective due to androgen action, some rats received an orchidectomy immediately following saporin injection (n = 6). The procedure is described in detail in Chapter 2. To summarize: following saporin injection, separate incisions were made in the scrotum and tunica. The testes, adipose tissue, and epididymis were removed and the testicular vasculature and spermatic cords ligated. Animals were given an s.c. injection of analgesic and placed in their home cages with access to running wheels.

Wheel revolutions were tracked daily to ensure that rats were engaging in exercise throughout the recovery period. A group of untreated and unexercised animals (n = 5) was included. Because some of the animals in the study were not included in all analyses due to histological or histochemical compromise, group sizes for each analysis are reported individually below (overall n = 50).

Histochemical and Histological Processing

Four weeks after saporin injection, animals were reanesthetized, and the left VL muscle was exposed and injected with BHRP (2 µL, 0.2%; Invitrogen, Carlsbad, CA), as described in Chapter 2. Animals were sacrificed, exsanguinated, and fixed (1% paraformaldehyde/1.25% glutaraldehyde). To confirm the specificity of the saporin injections, the VM and VL were dissected bilaterally and weighed. To confirm the effectiveness of castration, the bulbocavernosus and levator ani (BC/LA) muscles of the perineum were also dissected and weighed. The lumbar spinal cords were removed, post-fixed and cyroprotected overnight. Spinal cords were then embedded in gelatin, frozen, and sectioned transversely at 40 µm into four alternate series. One series was stained with thionin for use in cell counts, and the

remaining three series were immediately reacted to visualize BHRP using the tetramethyl benzidine protocol described in Chapter 2. Once reacted, sections were mounted on gelatin-coated slides, and counterstained with thionin.

Microscopy

Motoneuron Counts

Motoneuron counts were collected in order to confirm that CTB-saporin injection was effective in inducing motoneuron death. The method is more extensively detailed in Chapter 2. In summary, thionin-stained motoneurons in the left and right lateral motor columns were stereologically counted along the rostrocaudal distribution of BHRP-labeled motoneurons. Raw counts were corrected for sampling, and a ratio of the number of motoneurons in the left and right lateral motor columns was calculated in order to determine whether there were fewer motoneurons on the CTB-saporin injected left side when compared to the uninjected right side. This has proven to be a reliable indicator of assessing whether CTB-saporin successfully induced the death of motoneurons (Little et al., 2009; Cai et al., 2017; Chew et al., 2019).

Motoneuron counts were derived from a mean of 11.52 sections spaced 480 μm apart and distributed uniformly through the rostrocaudal extent of the quadriceps motoneuron pool range. This sampling scheme produced an average estimated coefficient of error (CE) of .058. (untreated, n = 5; SAP, n = 6; SAP+EXERCISE, n = 10; SAP+EXERCISE+CASTRATION n = 6).

Using similar methods, the number of BHRP-labeled motoneurons was assessed in all sections of the reacted series through the entire rostrocaudal extent of their distribution for all animals. Counts of BHRP-labeled quadriceps motoneurons were made under brightfield illumination, where somata could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed (untreated, n = 5; SAP, n = 6; SAP+EXERCISE, n = 6; SAP+EXERCISE+CASTRATION n = 5).

Motoneuron Morphometry

Measures of motoneuron morphometry were collected from one of the three series that underwent histochemical processing to visualize BHRP label. Motoneurons labeled with BHRP were counted in a similar fashion described above for cell counts in the non-histochemically reacted series. Measures of dendritic morphometry were collected by reconstructing BHRP-labeled dendritic arbors from individual sections and merging all reconstructed sections from a single animal into a single composite reconstruction. This composite was used to find the summed total length of labeled dendrites and analyze the radial distribution, radial extent, and rostrocaudal extent of dendritic label. These methods and their rationale are described in detail in Chapter 2 (untreated, n = 5; SAP, n = 6; SAP+EXERCISE, n = 6; SAP+EXERCISE+CASTRATION n = 6).

RESULTS

Running Performance

Animals ran consistently over the four weeks they were allowed access to running wheels, averaging 3.45 ± 0.40 (mean ± SEM) kilometers per day (Fig. 4.1). There was a group difference in daily distance run between gonadectomized and gonadally intact animals who received saporin [$t(15) = 3.67$, $p < 0.01$]. Overall, animals ran an average cumulative total of 101.96 ± 11.47 km over the four weeks of *ad lib* exercise.

Muscle Weights

Group difference in body weight were present [$F(3,24) = 12.30$, $p < 0.0001$], and thus raw muscle weights were corrected for body mass to assess potential effects of saporin, castration, and/or exercise on muscle weight (Fig. 4.2). In untreated animals, the corrected weights of the right (0.17 ±

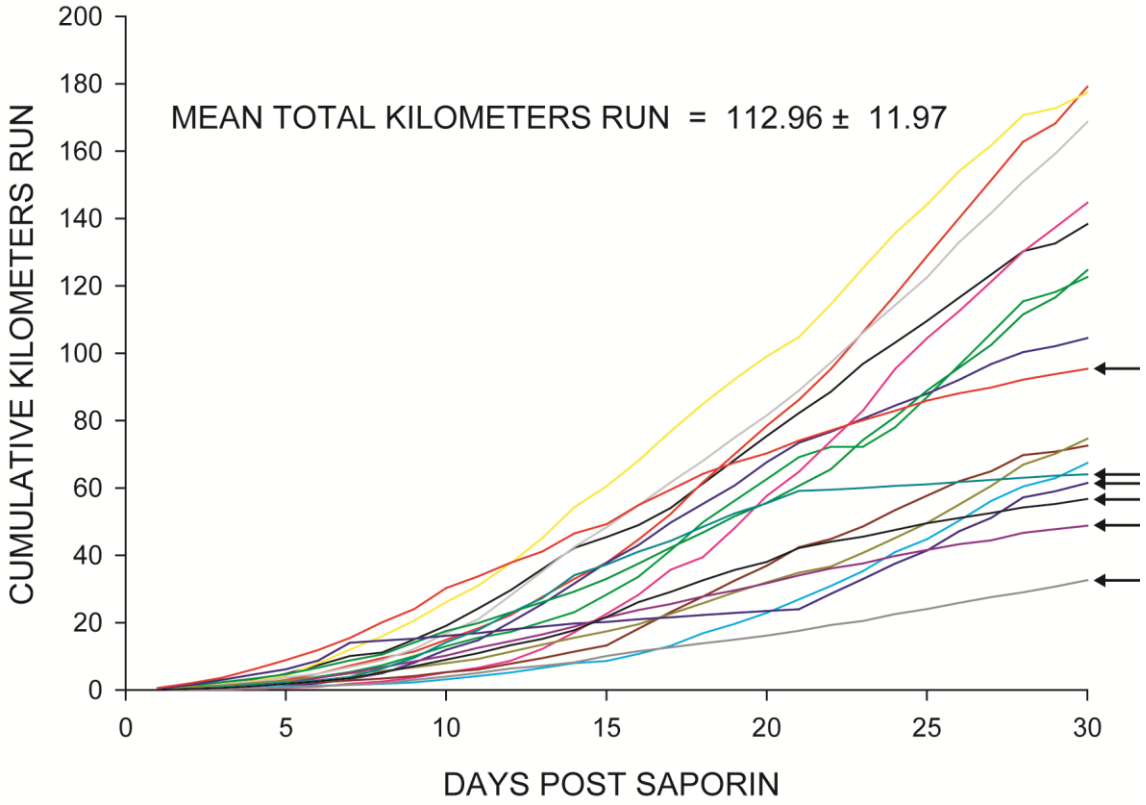


Figure 4.1. Total cumulative distance run in the 30 days following injection of saporin; each line represents the cumulative kilometers run by a single rat. Rats showed a fair amount of variation in both the total cumulative distance run and in the progressive increase in daily distance run. There was a group difference in the total cumulative distance run; arrows indicate rats that received an orchidectomy.

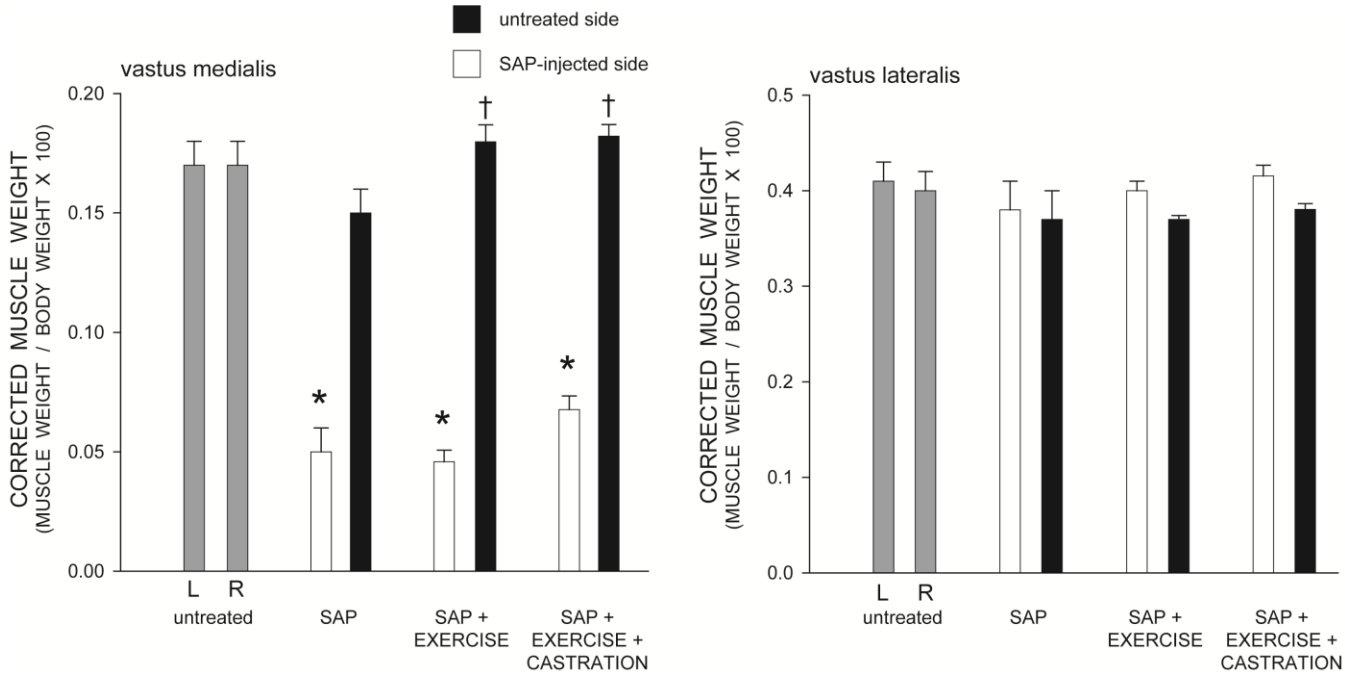


Figure 4.2. Weights of the vastus medialis muscles corrected by body weight in untreated animals, saporin-injected animals that either received no further treatment (SAP), were given *ad lib* exercise (SAP+EXERCISE), or were orchiectomized and given *ad lib* exercise (SAP+EXERCISE+CASTRATION), at four weeks after saporin injection. Gray bars represent weights from the right (R) and left (L) sides in untreated animals. Black bars represent weights from the untreated contralateral (right) side of the quadriceps muscle, and white bars represent weights from the saporin injected (left) side of the quadriceps muscle of saporin-injected animals. Saporin injection reduced the weight of the vastus medialis muscle. Exercise had no effect on saporin-injected muscle weight (left VM), but did have an effect on contralateral (right) vastus medialis weight. No group effects were present in either right or left vastus lateralis weights. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$).

0.01) and left (0.17 ± 0.01) VM muscles were similar [$t(4) = 2.14$, *ns*]. There was a significant effect of group on the weights of the uninjected (right) VM muscle [$F(3,24) = 3.34$, $p < 0.05$]; animals allowed to exercise had larger corrected muscle weights (0.18 ± 0.004) than animals not allowed to exercise [0.16 ± 0.008; average increase of 12.5%; LSDs, $p < 0.05$]. Uninjected VM weights across exercised groups did not differ from each other [LSDs, *ns*].

Injection of saporin into the left VM resulted in muscle atrophy in the saporin groups [overall average of 68% reduction in weight; $F(3 ,24) = 76.98$, $p < 0.0001$]. Compared to those of untreated animals, saporin-injected animals that received no further treatment had VM weights that were 74% lighter (LSD, $p < 0.0001$). Neither exercise nor castration prevented saporin-induced weight loss in the left VM; compared to those of untreated animals, saporin-injected rats who were allowed to exercise had VM weights that were 64% lighter (LSDs, $p < 0.0001$). Muscle weights across saporin groups did not differ from each other (LSDs, *ns*).

The effect of saporin injection on quadriceps weight was specific to the injected muscle. In untreated animals, the corrected weights of the right (0.40 ± .02) and left (0.41 ± .02) VL muscles were similar [paired t-test, $t(4) = .43$, *ns*]. The weights of the VL muscles on the untreated side did not differ across groups [$F(3,24) = .86$, *ns*]. Most importantly, the weights of the VL muscles adjacent of the saporin-injected VM muscles also did not differ across groups [$F(3,24) = .46$, *ns*].

BC/LA muscle weights from the present experiment were compared to previously collected data from Fargo and Sengelaub (2004a) in order to determine whether there were effects of saporin injection, castration, and/or exercise on BC/LA muscle weight (Fig. 4.3). As described above, group differences were present, and thus raw BC/LA weights were corrected for body mass to assess potential effects of saporin, exercise, and/or castration on muscle weight. There were group differences in corrected BC/LA muscle weights [$F(3,20) = 69.72$, $p < 0.0001$]. The corrected weight of the BC/LA in untreated animals

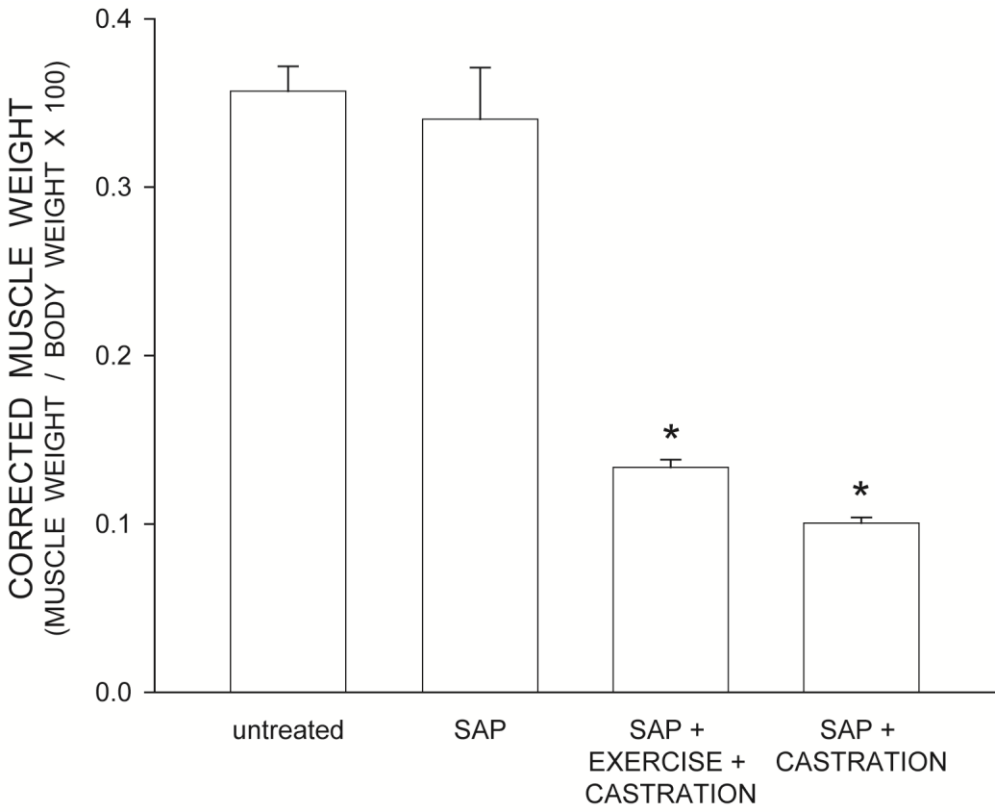


Figure 4.3. Weights of the bulbocavernosus and levator ani muscles corrected by body weight in untreated animals and saporin-injected animals that either received no further treatment (SAP), were orchiectomized prior to receiving saporin [SAP+CASTRATION; data collected by Fargo and Sengelaub (2004a,b)], or were orchiectomized and given *ad lib* exercise (SAP+EXERCISE+CASTRATION), at four weeks after saporin injection. Castration reduced the weight of the perineal musculature, and exercise had no effect on perineal muscle weight. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$).

(.36 ± .01) did not differ from those of gonadally intact animals who received saporin or gonadally intact animals allowed to exercise (LSDs, *ns*). Orchidectomy has a well-documented history of causing atrophy of the BC/LA (Kurz et al., 1986; Fargo and Sengelaub, 2004a), and this was replicated in the present study. Castrated exercised animals who received saporin showed reduced corrected BC/LA muscle weights (0.13 ± .00) compared to untreated animals (63% reduction; LSD, $p < 0.001$). Importantly, corrected BC/LA weights in castrated exercised animals who received saporin did not significantly differ from those of castrated sedentary animals who received saporin (LSD, *ns*).

Motoneuron Counts

In untreated animals, the number of motoneurons within the identified quadriceps range did not differ between the left (251.2 ± 14) and right (237.6 ± 25) motor column [paired t-test, $t(4) = 0.63$, *ns*]. Injection of saporin into the left VM resulted in the death of ipsilateral quadriceps motoneurons, significantly reducing the number of motoneurons in the left motor column relative to that of the right [$F(3,24) = 3.81$, $p < 0.03$; Fig. 4.4]. Unilateral injection of saporin into the left VM resulted in a 21% reduction in the relative number of motoneurons compared with that of untreated animals (LSD, $p < 0.02$). Neither exercise nor castration prevented the saporin-induced reduction in motoneuron number (overall average of 23% reduced; LSDs, $p < 0.001$ compared to untreated animals).

Motoneuron Morphometry

Injection of BHRP successfully labeled quadriceps motoneurons in all groups (Fig. 4.5). The dendritic arbor of labeled quadriceps motoneurons was strictly unilateral, with extensive ramification along the ventrolateral margins of the gray matter and in the lateral funiculus, as well as throughout the ventral horn. An average of 26.03 (± 1.56) motoneurons per animal was labeled with BHRP, and this did not vary across groups [$F(3,18) = 1.62$, *ns*].

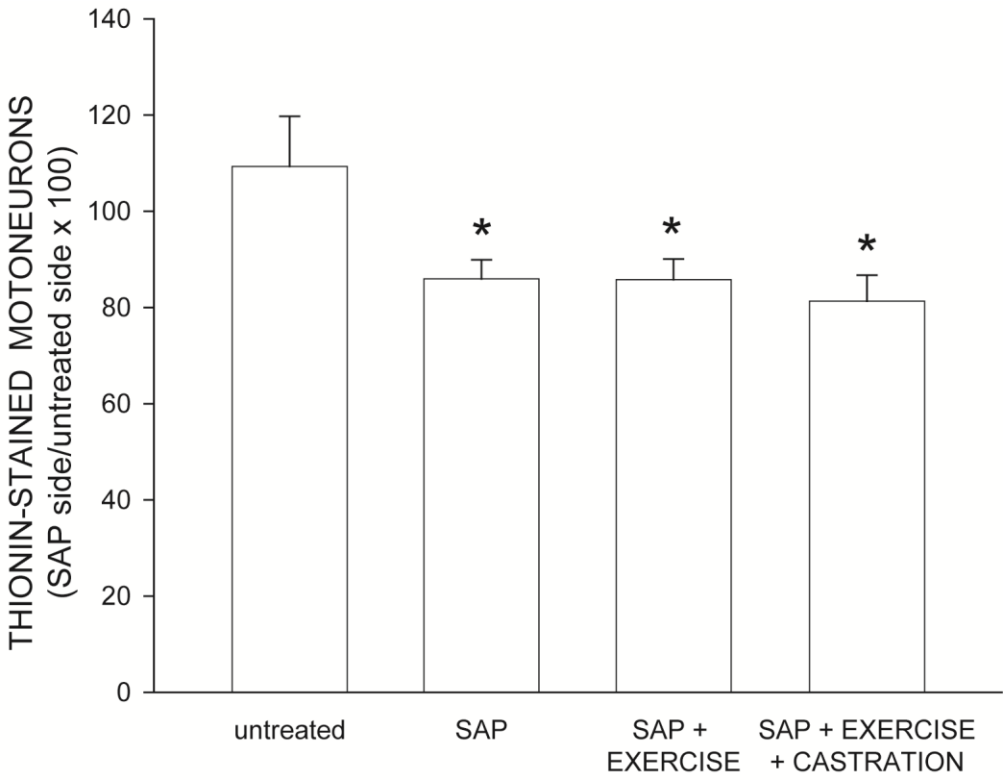


Figure 4.4. Numbers of quadriceps motoneurons in untreated animals, saporin-injected animals that either received no further treatment (SAP), were given *ad lib* exercise (SAP+EXERCISE), or were orchiectomized and given *ad lib* exercise (SAP+EXERCISE+CASTRATION), at four weeks after saporin injection, expressed as a ratio of motoneuron number ipsilateral to the saporin-injected muscle relative to that on the untreated side. Saporin killed approximately 21% of the ipsilateral quadriceps motoneurons, regardless of subsequent treatment. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$).

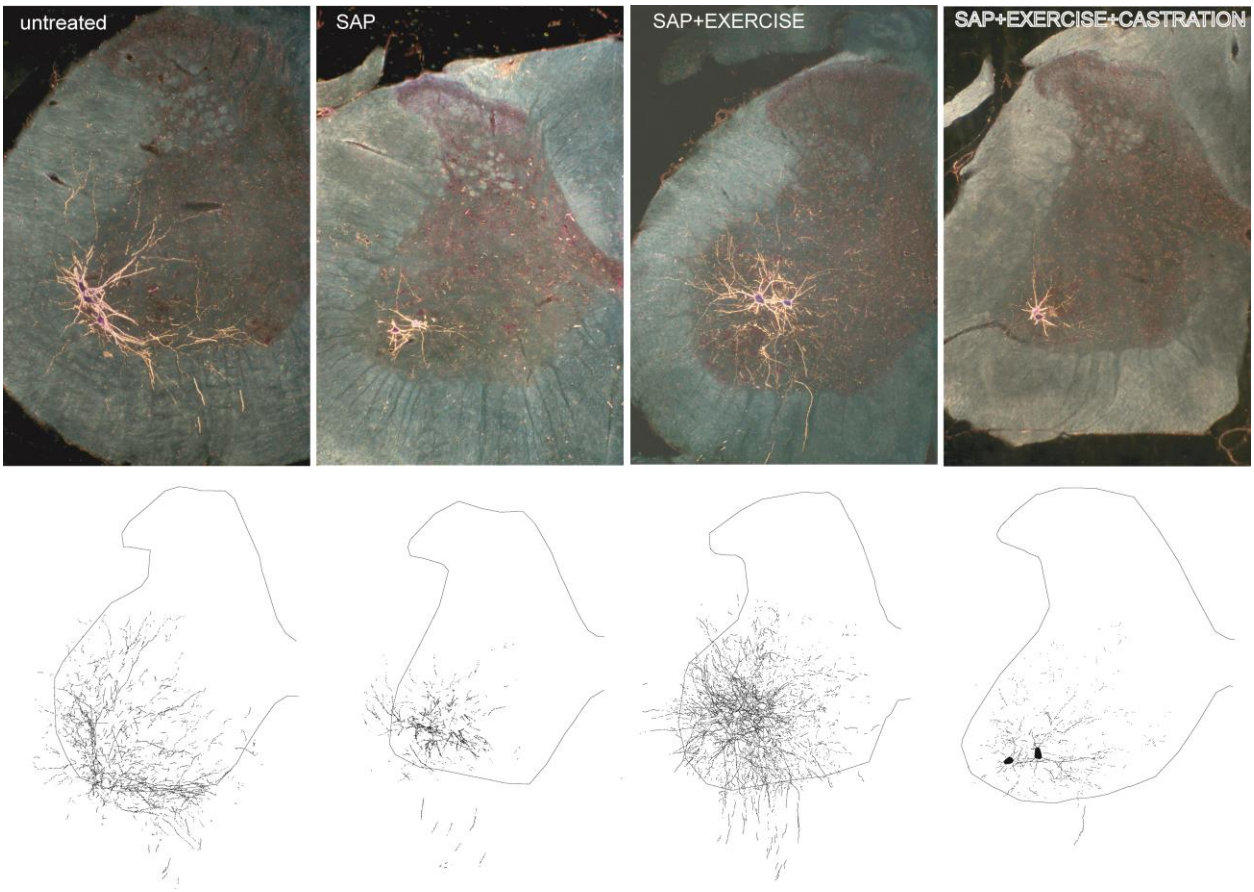


Figure 4.5. Darkfield digital micrographs of transverse hemisections through the lumbar spinal cords and computer-generated reconstructions BHRP-labeled somata and processes of an untreated animal (A,B) and saporin-injected animals with either no further treatment (C,D), *ad lib* exercise (E,F), or was orchiectomized and given *ad lib* exercise (G,H), after BHRP injection into the left vastus lateralis muscle. Computer-generated composites of BHRP labeling were drawn at 480 μm intervals through the entire rostrocaudal extent of the quadriceps motor pool; these composites were selected because they are representative of their respective group average dendritic lengths. Scale bar = 500 μm.

Dendritic Length

Surviving quadriceps motoneurons underwent marked dendritic atrophy (Fig. 4.6). Dendritic length was decreased by 64% in saporin-injected animals who received no further treatment compared to that of untreated animals [LSD, $p < .0001$; overall test for the effect of group on dendritic length $F(3,18) = 15.03$, $p < 0.0001$]. Compared to untreated animals, dendritic lengths were significantly shorter in all saporin-injected animals, regardless of exercise status or castration (LSDs, $p < 0.001$).

Exercise attenuated dendritic atrophy in saporin-injected animals, with dendritic length being reduced on average by only 28%. Saporin-injected animals who exercised had dendritic lengths were 97% longer than those without exercise (LSD, $p < 0.02$).

Castration prevented the attenuation of dendritic atrophy by exercise in saporin-injected animals. Dendritic lengths in exercised castrated saporin animals were significantly shorter (decreased by 45%) than those of gonadally intact exercised saporin animals (LSD, $p < 0.01$). Furthermore, dendritic lengths in castrated exercised saporin animals were not significantly different from those of saporin animals who received no exercise (LSD, *ns*).

Dendritic Distribution

Dendritic length was non-uniformly distributed across radial bins, and a repeated measures ANOVA revealed a significant effect of radial location [$F(11,198) = 9.64$, $p < 0.0001$; Fig. 4.7]. Consistent with the results seen in total dendritic length analysis, there was also a significant effect of group [$F(3,198) = 16.82$, $p < 0.0001$]. There were reductions in dendritic length throughout the radial distribution, ranging from 38% (180º to 240º) to 79% (60º to 120º) in saporin-injected animals compared with untreated animals [$F(1,99) = 26.19$, $p < .0007$]. Saporin-injected animals allowed to exercise showed attenuated reductions, with reductions in dendritic length ranging from no change (180º to 300º)

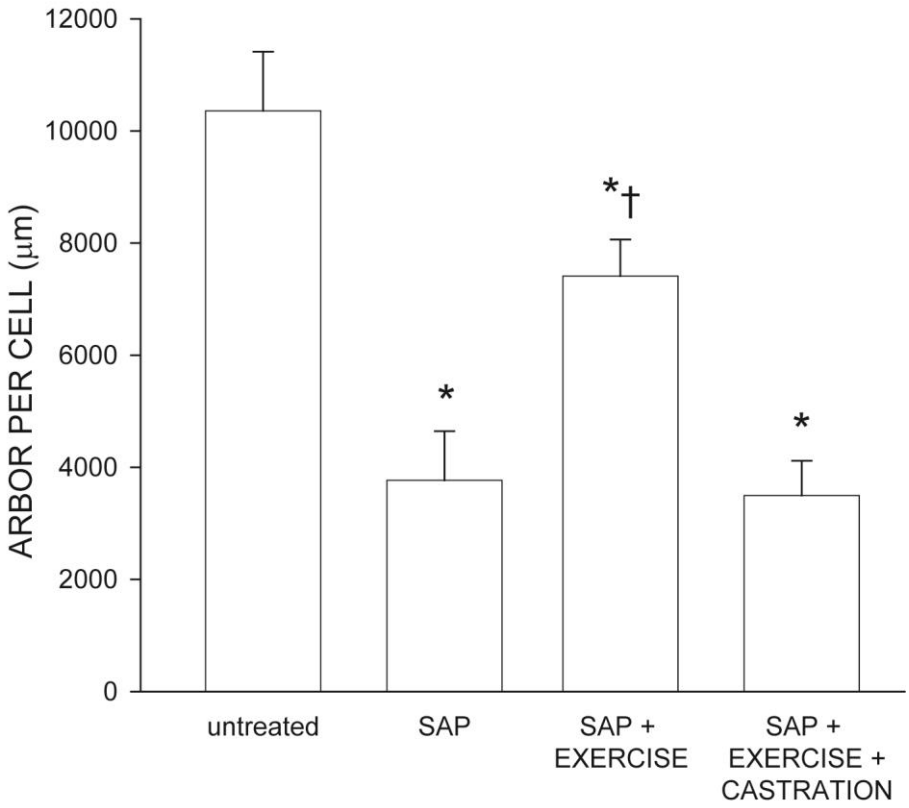


Figure 4.6. Dendritic lengths of quadriceps motoneurons in untreated animals, saporin-injected animals that either received no further treatment (SAP), were given *ad lib* exercise (SAP+EXERCISE), or were orchiectomized and given *ad lib* exercise (SAP+EXERCISE+CASTRATION). Following saporin-induced motoneuron death, surviving neighboring motoneurons lost almost 64% of their dendritic length. Exercise attenuated this dendritic atrophy, but castration prevented this attenuation. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$). † indicates significantly different from untreated saporin-injected animals ($p < 0.05$).

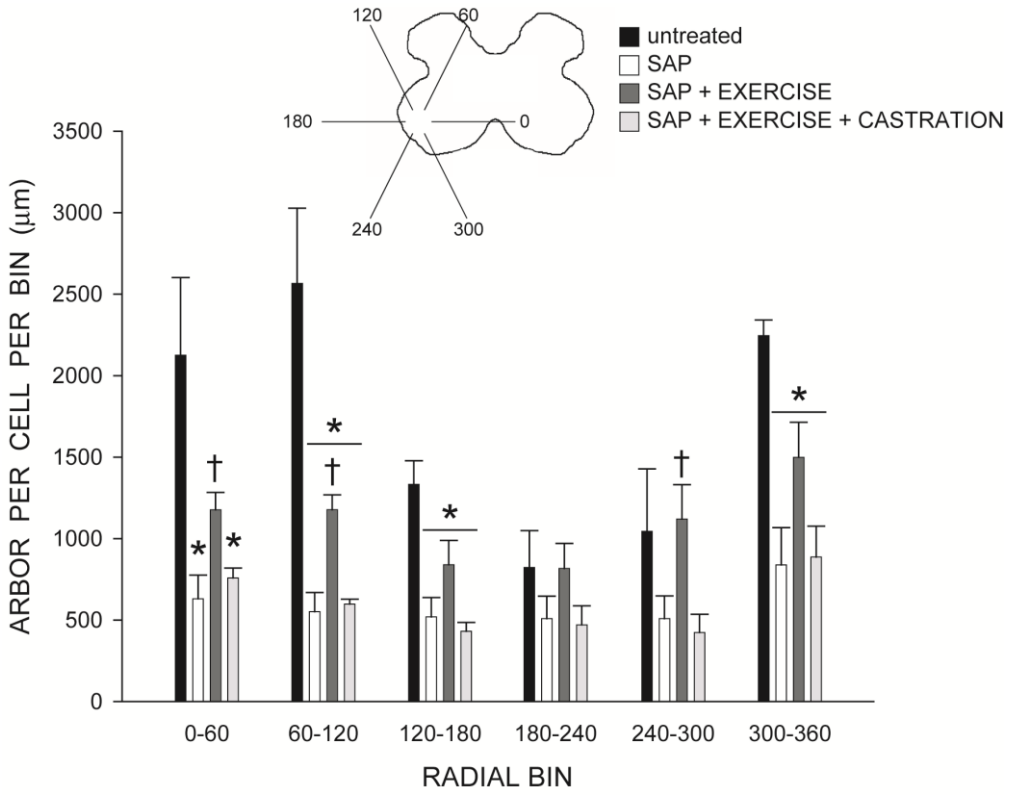


Figure 4.7. Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron dendritic distribution. Length per radial bin of quadriceps dendrites in untreated animals (black bars), saporin-injected animals that either received no further treatment (SAP; white bars), were given *ad lib* exercise (SAP+EXERCISE; dark gray bars), or were orchiectomized and given *ad lib* exercise (SAP+EXERCISE+CASTRATION; light gray bars). For graphic purposes, dendritic length measures have been collapsed into 6 bins of 60° each. Quadriceps motoneuron dendritic arbors display a non-uniform distribution, with the majority of the arbor located between 300° and 120°. Following saporin-induced motoneuron death, surviving neighboring motoneurons had reduced dendritic length throughout the radial distribution. Exercise attenuated this reduction, and castration prevented the attenuation. Bar heights represent means ± SEM. * indicates significantly different from untreated animals ($p < 0.05$). † indicates significantly different from untreated saporin-injected animals ($p < 0.05$).

to only 54% (60º to 120º) compared to untreated animals [$F(1,99) = 9.44$, $p < .02$]. Throughout most of the radial distribution, dendritic lengths per bin in exercised saporin-injected animals were longer than those of saporin-injected animals who received no further treatment [$F(1,110) = 9.70$, $p < 0.02$], with increases ranging from 61% (180º-240º) to 120% (240º-300º).

Castrated exercised saporin animals showed reductions in dendritic length ranging from 43% (180º to 240º) to 77% (60º to 120º) compared to untreated animals [$F(1,88) = 33.92$, $p < 0.001$], and these reductions were not significantly different from those seen in saporin animals who received no further treatment [$F(1,99) = 0.00$, *ns*]. Dendritic lengths per bin in castrated exercised saporin animals were shorter than those of gonadally intact exercised saporin animals [$F(1,99) = 16.39$, $p < 0.01$], with reductions ranging from 35% (0º to 60º) to 62% (240º to 300º).

Dendritic Extent

In agreement with the nonuniform dendritic distribution of quadriceps motoneurons (Fig. 4.7), radial extent differed across bins (Fig. 4.8), and a repeated measures ANOVA revealed a significant effect of radial bin [$F(11,198) = 21.46$, $p < 0.0001$]. There was not a significant effect of group [$F(3, 198) = 2.51$, *ns*] in dendritic extent.

Rostrocaudal dendritic extent spanned 3776.0 ± 546.3 µm in untreated animals. Neither saporin injection, exercise, nor castration had an effect on rostrocaudal extent [overall average 3731 ± 177.2 µm; $F(3,18) = .01$, *ns*].

DISCUSSION

Surviving motoneurons respond to the death of neighboring motoneurons with marked dendritic atrophy (Little et al., 2009). Treatment with testosterone, mediated by classical receptor activation (Cai

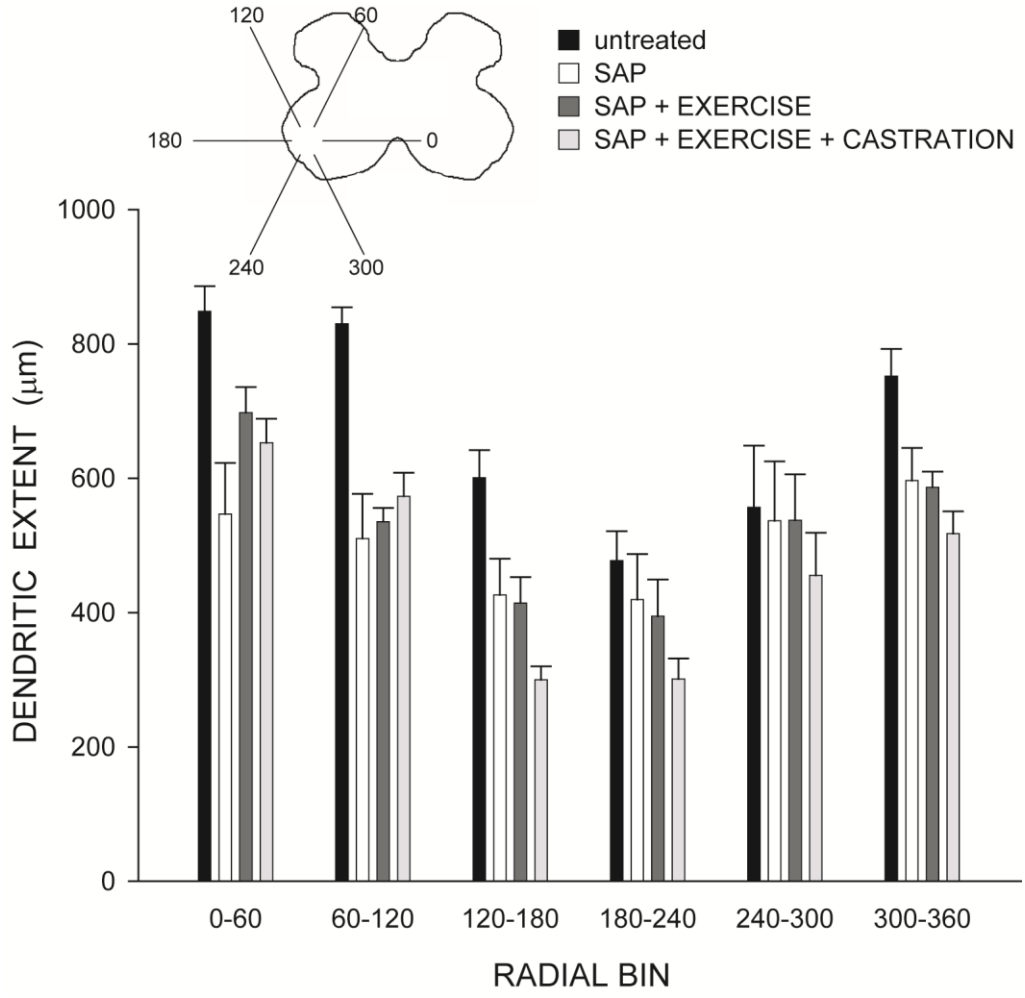


Figure 4.8. Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron radial dendritic extent. Radial extents of quadriceps dendrites in untreated animals (black bars), saporin-injected animals that either received no further treatment (SAP; white bars), were given *ad lib* exercise (SAP+EXERCISE; dark gray bars), or were orchiectomized and given *ad lib* exercise (SAP+EXERCISE+CASTRATION; light gray bars). For graphic purposes, dendritic extent measures have been collapsed into 6 bins of 60° each. Extent measures did not differ across groups. Bar heights represent means ± SEM.

et al., 2017), or exercise (Chew and Sengelaub, 2019) are protective against this atrophy. In this chapter, I have demonstrated that the neuroprotective effects of exercise are dependent on the presence of testes, likely through their secretion of androgens (Chew and Sengelaub, 2020).

Running wheel performance

Consistent with prior results, saporin injection did not affect the ability of the rats to exercise. Running wheel performance in saporin-treated animals did not differ from that of intact animals, and gonadally intact exercised saporin animals ran an overall cumulative distance of roughly 125 km over the four weeks of treatment.

Castration did have an effect on rats' running, both in daily distance run and total cumulative distance over the four weeks following saporin injection and orchidectomy. Gonadally intact rats typically showed an initial interest in their newfound access to their running wheels; some animals began running as soon as five minutes following recovery from anesthesia. Daily distance run increased throughout the four weeks of *ad lib* exercise, which is likely due to a combination of initial habituation to a new environment and a progressive gain of fitness as running behavior continued over time. In contrast, castrated rats showed an initial reluctance to engage in running, which I believe was due to post-operative discomfort following the more invasive orchidectomy surgical procedure compared to rats who only received saporin injection.

Despite the initial poor performance in daily distance run, castrated rats did show the normal increase in daily distance run as more time passed post-surgery. During the second week following saporin injection and orchidectomy, castrated rats showed increases in daily distance run that mirrored the increases seen in daily distance run of gonadally intact rats from their first week following surgery. By time of sacrifice, **four** weeks following surgery, the cumulative distance run by castrated rats resembled the cumulative distance run by gonadally intact rats **three** weeks following initial surgery

[$F(3,36) = 1.67$, *ns*]. This suggests that castrated rats show seemingly normal progression of increases in daily distance run, albeit on a shifted time scale to allow for recovery from a more invasive surgery. Because a large component of cumulative distance run occurs in the final week prior to sacrifice, the delay in the ramping of running behavior in castrated rats is the likely cause of the decrease in cumulative distance run.

Previous work from our lab indicates that the effective temporal window for attenuation of dendritic atrophy with androgen treatment occurs in the final two weeks of a four week recovery period (Coons and Sengelaub, 2008). In other words, delaying androgen treatment by two weeks following saporin injection is still sufficient to provide neuroprotection. If my hypothesis that exercise utilizes an androgen-dependent mechanism is correct, then the reduction in running during the first week following orchidectomy would not be expected to affect the ability of exercise to provide neuroprotection. Thus, the failure of exercise to protect motoneuron dendrites in castrated animals can be attributed to the lack of androgens, rather than a reduction in running following injury. This is further corroborated by the similarity of the dendritic morphology in castrated rats and rats who received local androgen receptor blockade at the quadriceps (reported in Chapter 5).

Furthermore, while there was an overall group difference in cumulative distance run between castrated and gonadally intact rats, the cumulative distance run by all castrates was comparable to the low end of the distribution of distances run by gonadally intact animals (Fig. 4.1). While not conclusive evidence when taken alone, this does suggest that the lower average total cumulative distance run is not currently a limiting factor in the ability of exercise to protect motoneuron dendrites in castrates.

Saporin Injection and Quadriceps Muscle Weights

Similar to the results discussed in Chapter 3, saporin injection into the VM reduced muscle weight and the number of innervating motoneurons. This induced death was specific to the motoneurons

innervating the saporin-injected VM muscle; there were no changes in the number of BHRP-labeled motoneurons projecting to the adjacent VL. This remains an important consideration for interpreting the effects seen on the morphology of surviving motoneurons. The unchanged number of motoneurons innervating the VL indicates that the changes in their dendritic morphology we observed (see below) cannot be due to accidental spread of saporin in the periphery (and subsequent death of VL motoneurons).

Neither exercise nor castration prevented saporin-induced decreases in the weight of the injected VM muscle, nor did they prevent saporin-induced motoneuron death. Thus, the beneficial effects of exercise on the morphology of neighboring surviving motoneurons, and the occlusion of these benefits with castration, cannot be attributed to differences resulting from the degree of peripheral damage or an attenuation of the ability of saporin to kill motoneurons.

One notable deviation from prior results is that animals allowed to exercise, regardless of castration, showed a modest increase in the weight of the uninjected (right) VM. This hypertrophy could be an exercise-dependent response to the saporin-induced reduction of the left VM, with the right VM compensating for the contralateral reduction in force production and weight bearing of the left VM (Tsumiyama et al., 2014). It is also interesting to note that this hypertrophy also occurred in castrated animals that have been deprived the major source of endogenous anabolic steroids, although local steroidogenesis in skeletal muscle has been reported in the past (Aizawa et al., 2007; 2008, 2010). If local steroidogenesis is occurring within skeletal muscle, then it is possible that locally produced androgens could drive muscular hypertrophy, thus not requiring gonadal androgen production. If local steroidogenesis is insufficient to produce muscular hypertrophy, then it is likely that the observed hypertrophy is the result of a non-androgenic mechanism.

Effectiveness of Castration on Androgen Production

Castration was effective in reducing systemic circulation of androgens, which was confirmed by comparing weights of the BC/LA across castrated and non-castrated groups. The weights of the BC/LA in castrated animals were reduced by ~63% compared to those of saporin-injected or untreated animals, and this atrophy was not affected by exercise.

The SNB and innervated BC/LA are an androgen-sensitive system (Breedlove and Arnold, 1980, 1983c). Androgens play a critical role in the initial masculinization of the neuromuscular complex during development (Breedlove et al., 1982; Breedlove and Arnold, 1983b), and the complex remains sensitive to androgen manipulation into adulthood (Kurz et al., 1986; Rand and Breedlove, 1995). Specifically, castration of adult male rats causes atrophy of perineal muscle mass (Wainman and Shipounoff, 1941) and testosterone replacement prevents said atrophy (Kurz et al., 1986; Fargo and Sengelaub, 2004a,b). Administration of exogenous testosterone to gonadally intact rats has also been demonstrated to cause hypertrophy of the BC/LA (Wainman and Shipounoff, 1941). Together, these findings indicate that the mass of the BC/LA is sensitive to androgen deprivation, replacement, and supplementation, making it a reliable metric for assessing circulating androgen titers.

While the testes are the major source of androgen production in males, it is possible that exercise could cause an upregulation of alternative sources of androgen production. For example, the adrenal gland produces androgens that are systemically circulated, albeit in low concentrations compared to the testes, and there have been documented instances of exercise inducing steroidogenesis locally in skeletal muscle (Aizawa et al., 2010). However, the data from the present study indicates that exercise was not able to prevent the atrophy of the BC/LA following castration, eliminating the possibilities that exercise maintains or upregulates systemic androgen concentrations or causes sufficient local steroidogenesis to maintain androgen sensitive musculature in castrated animals.

Androgen-Dependent Protection from Dendritic Atrophy

Saporin-induced motoneuron death resulted in a pronounced dendritic atrophy in surviving quadriceps motoneurons (Little et al., 2009). As previously mentioned, we have ruled out the loss of afferent fibers from the saporin-injected muscle (Cai et al., 2017) or the increase in activated microglia in the quadriceps motor pool following saporin-induced motoneuron death (Chew et al., 2019) as causes of dendritic atrophy. Our lab has proposed that the induced death of motoneurons could result in the release of toxins (see Chapter 3) that damage the dendrites of surviving motoneurons into the extracellular space (Fargo and Sengelaub, 2004a,b; Chew et al., 2019). Such local changes in the cellular microenvironment would be consistent with the general atrophy seen throughout the dendritic distribution, and both exercise and testosterone upregulate expression of factors that could mitigate damage caused by such toxins (see Chapter 3).

The present study provides compelling evidence that the neuroprotective effects of exercise are dependent on androgen action. These results are consistent with similar findings of the importance of gonadal hormones in neuroprotective mechanisms (Kujawa et al., 1989; 1991; Fargo and Sengelaub, 2004a,b; Little et al., 2009; Thompson et al., 2014). The presence of androgens has also been demonstrated to be necessary for the neurotherapeutic effects of exercise in promoting axonal regeneration following axotomy; castrated rats allowed to exercise show significantly fewer axon sprouts from the distal stump of the regenerating axon compared to gonadally intact rats that undergo the same exercise therapy (Thompson et al., 2014).

Exercise results in elevations in serum testosterone (Kindermann et al., 1982; Wood et al., 2012; Sato and Iemitsu, 2015), although intensity, duration, prior conditioning, time point of measurement (e.g., immediately vs. hours after exercise; Vingren et al., 2010), or type of training (Tremblay et al., 2004; Sato and Iemitsu, 2015) all contribute to how testosterone concentrations change in response to

exercise. Despite a lack of clarity as to how testosterone concentrations change in response to exercise, the results from the present study indicate that the interaction between exercise and testosterone production is likely necessary in order to protect motoneuron dendrites from collateral dendritic atrophy. It is possible that exercise causes an upregulation in testosterone production, which is then protective to motoneuron dendrites in a manner that mirrors exogenous supplementation of testosterone, as demonstrated by Little et al. (2009).

It is also possible that it is not the **upregulation** of androgens that is necessary for exercise-driven neuroprotection, but rather that normal circulating concentrations of androgens are necessary to contribute to some alternative exercise-driven mechanism that is neuroprotective. Similarly, the results of the present experiment could indicate that an effect of the orchidectomy that is not related to androgens ablated the previously observed protective effect of exercise on surviving motoneuron dendrites. An experiment using the same model of partial motoneuron depletion in a group that is castrated, allowed to exercise, and also receives androgen replacement could provide more powerful evidence that lack of exercise-driven neuroprotection in castrates is due to lack of available androgens. If exercise is unable to protect surviving motoneuron dendrites in a castrated group that also receives androgen replacement, then this would indicate that a non-androgenic effect of castration contributes to exercise-driven neuroprotection. However, in light of the results of Chapters 5 and 6 of this dissertation, this hypothesis seems unlikely and will be addressed in Chapter 8.

CONCLUSIONS

Chapter 3 of this thesis has established that exercise is neuroprotective on motoneuron dendrites following the induced death of their neighbors (Chew and Sengelaub, 2019). Here we tested whether the neuroprotective effect of exercise is dependent on the presence of androgens. Our findings indicate that

castration prevents neuroprotection by exercise, and that androgens are necessary for exercise-mediated neuroprotection of motoneurons following the death of their neighbors (Chew and Sengelaub, 2020). These findings provide strong evidence that exercise is neuroprotective to motoneuron dendrites via a hormonal mechanism, but are not able to determine how or where androgens must act in order to protect motoneuron dendrites. These specifics of the mechanism underlying exercise treatment are addressed in Chapter 5.

CHAPTER 5

IS NEUROPROTECTION OF MOTONEURON DENDRITES BY EXERCISE DEPENDENT ON ANDROGEN ACTION AT THE TARGET MUSCLE?

This chapter is currently in press as Chew, C., & Sengelaub, D.R. (2020). Exercise is neuroprotective on the morphology of somatic motoneurons following the death of neighboring motoneurons via androgen action at the target muscle. *Development Neurobiology.*

We have established that the loss of motoneuron has adverse effects on the structure and function of surviving motoneurons using a rat model of motoneuron death, including dendritic atrophy and a resulting decrease in electrophysiological excitability (Little et al., 2009; Cai et al., 2017). This induced atrophy and decrease in excitability is responsible for at least some of the movement deficits that accompany disease or injury-related loss of motoneurons. Given that we currently lack the technology to replace dead motoneurons, protecting surviving motoneurons from injury-induced atrophy is an important goal.

Treatment with gonadal steroids is neuroprotective in a variety of contexts (Foecking et al., 2015), and it has been specifically established that treatment with exogenous testosterone is protective against induced dendritic atrophy and reduced excitability following the death of adjacent motoneurons (Fargo and Sengelaub, 2004a,b; Little et al., 2009). This effect of androgens is mediated via classical receptor activation, and systemic blockade of androgen receptors completely prevents the neuroprotective effects (Cai et al., 2017). This suggests that receptor action is a necessary driver of the neuroprotective benefits of androgens.

We have also shown that *ad lib* exercise is neuroprotective against dendritic atrophy, to a degree comparable to that seen when rats are treated with testosterone (Chew and Sengelaub, 2019). Many of the adaptive effects seen in muscle following exercise have been linked to androgen signaling pathways (Bhasin et al., 2001a, 2003; Aizawa et al., 2010; Hedayatpour and Falla, 2015). Administration of supraphysiological dosages of testosterone combined with exercise has been shown to increase lean muscle mass and muscle cross sectional area in human males (Bhasin et al., 1996, 2001a). Exercise with supplementary testosterone alters skeletal muscle synthesis and oxidation of glycogen compared to similarly exercised rats who did not receive testosterone (van Breda et al., 1993). Exercise accelerates axon sprouting and regeneration following axotomy via an androgen-dependent manner (Thompson et al., 2014). The similarity between the neuroprotective efficacy of exercise and exogenous testosterone treatment, the preexisting links between androgens and exercise adaptations, and the previously reported androgen dependency of exercise on other neurotherapeutic effects leads us to believe that the neuroprotective effects of exercise may be dependent on androgen action.

Furthermore, neurons and their innervated targets have been shown to have coregulatory effects on each other. Skeletal muscles express gonadal hormone receptors (Dubé et al., 1976), and hormonal manipulations at the muscle can cause changes in protein content in both the muscle and innervating motoneurons (Verhovshek and Sengelaub, 2013), number of motoneurons (Fishman and Breedlove, 1992), or the morphology; (Kurz et al., 1986; Rand and Breedlove, 1995; Huguenard et al., 2011) of the innervating motoneurons. Perhaps most importantly, it has been demonstrated that attenuation of dendritic atrophy in motoneurons following the death of their neighbors with testosterone treatment is dependent on androgen receptor action at the site of the target muscle (Chung, 2015). Taken together, this seems to indicate that there is a well-established mechanism for hormones modulating motoneuron morphology via target-dependent androgen action, either following injury or in normal maintenance of certain neuronal structures.

However, it remains unclear whether this mechanism can be extrapolated to include neuroprotection via exercise. Thus, the studies in this chapter test the following questions: 1) do the neuroprotective effects of exercise following partial motoneuron depletion depend on androgen action, and 2) if so, are the androgen-dependent neuroprotective effects of exercise driven by androgen receptor action at the muscle?

METHODS AND DESIGN

Animals

Adult male Sprague-Dawley rats (Envigo, Indianapolis, IN) approximately 100 days old were used for this experiment. We used the toxin saporin, conjugated to the cholera toxin B subunit, to kill motoneurons, as described in Chapter 2. Briefly, rats were anesthetized with isoflurane, the left vastus medialis (VM) muscle was exposed and injected with CTB-saporin (2 µL, 0.1%; Advanced Targeting Systems, Inc., San Diego, CA).

Some rats were not treated further (n = 6), whereas others were immediately allowed free access to exercise wheels (width = 11.2 cm; diameter = 37 cm; circumference = 116 cm) attached to their home cages (n = 10). To assess whether exercise is neuroprotective due to androgen action at the muscle, additional groups of saporin-injected, gonadally intact, exercised rats were treated with the nonsteroidal androgen receptor antagonist hydroxyflutamide (hFlut; 2-hydroxy-flutamide; LKT Laboratories, St. Paul, MN) immediately after saporin injection and prior to placement in their cages. Some saporin-injected rats had Silastic implants (12.5 x 3.5 x 1.5 mm) impregnated with hydroxyflutamide (0.2 mg) sutured onto the left vastus lateralis (VL) muscle (n=12). Another group of saporin-injected, exercised

rats were given identical implants placed subcutaneously in the interscapular area to control for potential systemic effects (n=17).

Wheel revolutions were tracked daily to ensure that rats were engaging in exercise throughout the recovery period. A group of untreated and unexercised animals (n = 5) was included. Because some of the animals in the study were not included in all analyses due to histological or histochemical compromise, group sizes for each analysis are reported individually below (overall n = 50).

Histochemical and Histological Processing

Four weeks after saporin injection, animals were reanesthetized, and the left VL muscle was exposed and injected with BHRP (2 µL, 0.2%; Invitrogen, Carlsbad, CA), as described in Chapter 2. Animals were sacrificed, exsanguinated, and fixed (1% paraformaldehyde/1.25% glutaraldehyde). To confirm the specificity of the saporin injections, the VM and VL were dissected bilaterally and weighed, and the lumbar spinal cords were removed and prepared for histochemical processing. Spinal cords were post-fixed and cyroprotected overnight, then embedded in gelatin, frozen, and sectioned transversely at 40 µm into four alternate series. One series was stained with thionin for use in cell counts, and the remaining three series were immediately reacted to visualize BHRP using the tetramethyl benzidine protocol described in Chapter 2. Once reacted, sections were mounted on gelatin-coated slides, and counterstained with thionin.

Microscopy

Motoneuron Counts

Motoneuron counts were collected in order to confirm that CTB-saporin injection was effective in inducing motoneuron death. The method is more extensively detailed in Chapter 2. In summary, thionin-stained motoneurons in the left and right lateral motor columns were stereologically counted

along the rostrocaudal distribution of BHRP-labeled motoneurons. Raw counts were corrected for sampling, and a ratio of the number of motoneurons in the left and right lateral motor columns was calculated in order to determine whether there were fewer motoneurons on the CTB-saporin injected left side when compared to the uninjected right side. This has proven to be a reliable indicator of assessing whether CTB-saporin successfully induced the death of motoneurons (Little et al., 2009; Cai et al., 2017; Chew et al., 2019).

Motoneuron counts were derived from a mean of 10.84 sections spaced 480 µm apart and distributed uniformly through the rostrocaudal extent of the quadriceps motoneuron pool range. This sampling scheme produced an average estimated coefficient of error (CE) of .061. (untreated, n = 5; SAP, n = 6; SAP+EXERCISE, n = 10; SAP+EXERCISE+hFlut MUS, n = 12; SAP+EXERCISE+hFlut SCAP, n = 17).

Using similar methods, the number of BHRP-labeled motoneurons was assessed in all sections of the reacted series through the entire rostrocaudal extent of their distribution for all animals. Counts of BHRP-labeled quadriceps motoneurons were made under brightfield illumination, where somata could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed (untreated, n = 5; SAP, n = 6; SAP+EXERCISE, n = 6; SAP+EXERCISE+hFlut MUS, n = 9; SAP+EXERCISE+hFlut SCAP, n = 10).

Motoneuron Morphometry

Measures of motoneuron morphometry were collected from one of the three series that underwent histochemical processing to visualize BHRP label. Motoneurons labeled with BHRP were counted in a similar fashion described above for cell counts in the non-histochemically reacted series. Measures of dendritic morphometry were collected by reconstructing BHRP-labeled dendritic arbors from individual sections and merging all reconstructed sections from a single animal into a single

composite reconstruction. This composite was used to find the summed total length of labeled dendrites and analyze the radial distribution, radial extent, and rostrocaudal extent of dendritic label. These methods and their rationale are described in detail in Chapter 2 (untreated, n = 5; SAP, n = 6; SAP+EXERCISE, n = 6; SAP+EXERCISE+hFlut MUS, n = 9; SAP+EXERCISE+hFlut SCAP, n = 10).

RESULTS

Running Performance

Animals ran consistently over the four weeks they were allowed access to running wheels, averaging 4.26 ± 0.25 (mean ± SEM) kilometers per day (Fig. 5.1). Injection of saporin or delivery of hydroxyflutamide implants had no effect on exercise, and daily distance run in animals allowed to run did not differ across groups [$F(2, 32) = .85$, *ns*]. Overall, animals ran an average cumulative total of 126.59 ± 7.40 km over the four weeks of *ad lib* exercise.

Muscle Weights

Group difference in body weight were present [$F(4,47) = 12.94$, $p < 0.0001$], and thus raw muscle weights were corrected for body mass to assess potential effects of saporin, hormone treatment, and/or exercise on muscle weight (Fig. 5.2). In untreated animals, the corrected weights of the right (0.17 ± 0.01) and left (0.17 ± 0.01) VM muscles were similar [$t(4) = 2.14$, *ns*]. There was a significant effect of group on the weights of the uninjected (right) VM muscle [$F(4,47) = 6.61$, $p < 0.001$]; animals allowed to exercise had larger corrected muscle weights (0.19 ± 0.002) than animals not allowed to exercise [0.16 ± 0.008; average increase of 18%; LSDs, $p < 0.05$]. Uninjected VM weights across exercised groups did not differ from each other [LSDs, *ns*].

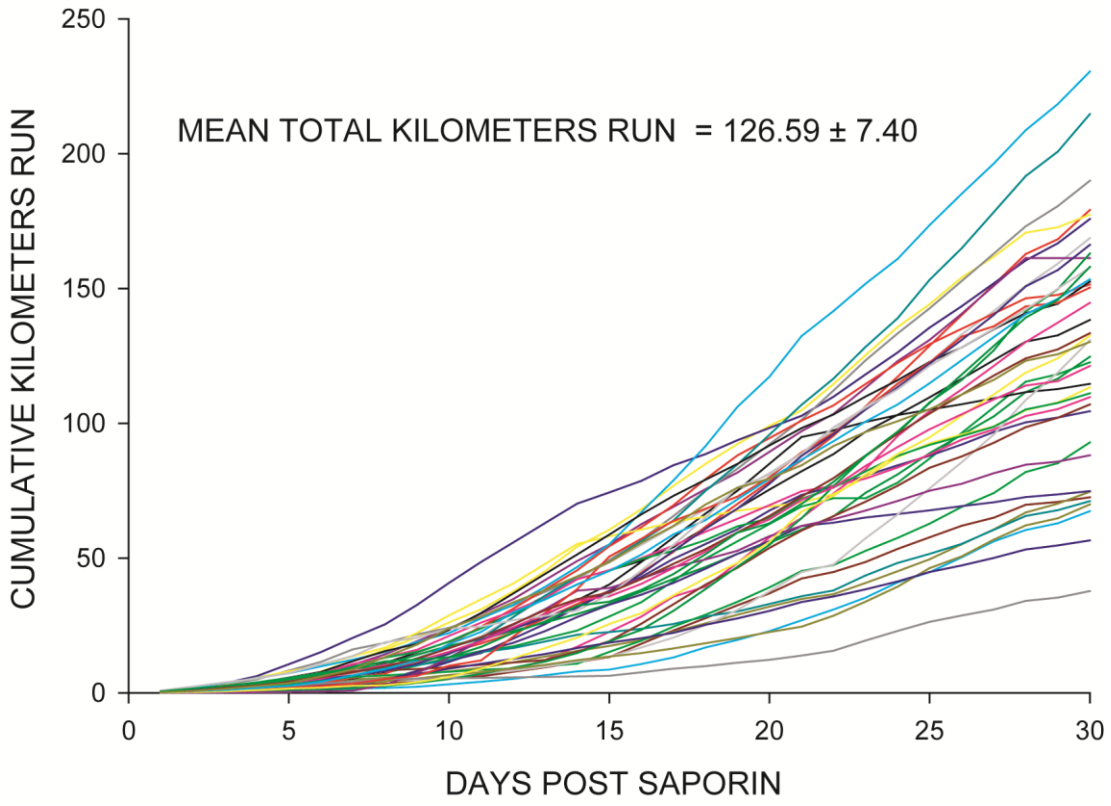


Figure 5.1. Total cumulative distance run in the 30 days following injection of saporin; each line represents the cumulative kilometers run by a single rat. Rats showed a large amount of variation in both the total cumulative distance run and in the progressive increase in daily distance run. However, there were no group differences in either total cumulative distance or daily distance run.

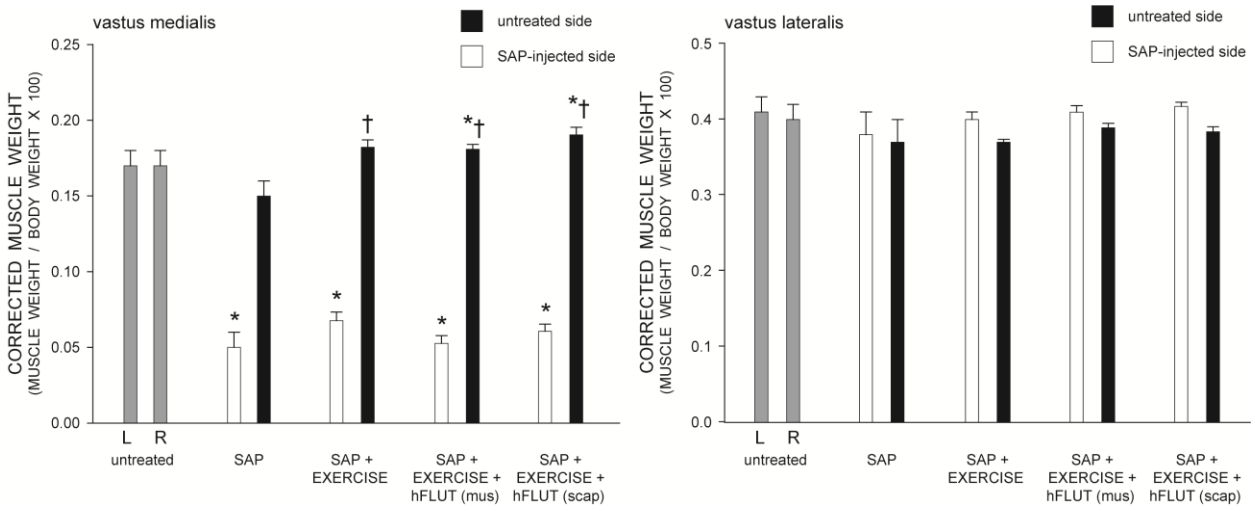


Figure 5.2. Weights of the vastus medialis and vastus lateralis muscles corrected by body weight in untreated animals and saporin-injected animals that either received no further treatment (SAP), were given *ad lib* exercise (SAP+EXERCISE), or were given *ad lib* exercise and received a hydroxyflutamide implant at either the ipsilateral vastus lateralis [SAP+EXERCISE + hFLUT (mus)] or scapula [SAP+EXERCISE + hFLUT(scap)], at four weeks after saporin injection. Gray bars represent weights from the right (R) and left (L) sides in untreated animals. Black bars represent weights from the untreated contralateral (right) leg, and white bars represent weights from the saporin injected (left) leg of saporin-injected animals. Saporin injection reduced the weight of the injected vastus medialis muscle; neither exercise nor androgen receptor blockade had an effect on left vastus medialis weight in saporin injected animals. Saporin animals who exercised showed minor hypertrophy of the (right) vastus medialis contralateral to the saporin injected muscle. Weights of both the left and right vastus lateralis were not affected by saporin injection or exercise. Bar heights represent means ± SEM. * indicates significantly different from untreated animals. † indicates significantly different from untreated saporin-injected animals.

Injection of saporin into the left VM resulted in muscle atrophy in the saporin groups [overall average of 68% reduction in weight; $F(4,47) = 43.80$, $p < 0.0001$]. Compared to those of untreated animals, saporin-injected animals that received no further treatment had VM weights that were 74% lighter (LSD, $p < 0.0001$). Exercise did not prevent saporin-induced weight loss in the left VM; compared to those of untreated animals, saporin-injected rats who were allowed to exercise had VM weights that were 64% lighter (LSD, $p < 0.0001$). Muscle weights across saporin groups did not differ from each other [$F(3,43) = 1.37$, *ns*].

The effect of saporin injection on quadriceps weight was specific to the injected muscle. In untreated animals, the corrected weights of the right (0.40 ± .02) and left (0.41 ± .02) VL muscles were similar [paired t-test, $t(4) = .43$, *ns*]. The weights of the VL muscles on the untreated side did not differ across groups [$F(4,47) = 1.27$, *ns*]. Most importantly, the weights of the VL muscles adjacent of the saporin-injected VM muscles also did not differ across groups [$F(4,47) = .95$, *ns*].

Motoneuron Counts

In untreated animals, the number of motoneurons within the identified quadriceps range did not differ between the left (251.2 ± 14) and right (237.6 ± 25) motor column [paired t-test, $t(4) = 0.63$, *ns*]. Injection of saporin into the left VM resulted in the death of ipsilateral quadriceps motoneurons, significantly reducing the number of motoneurons in the left motor column relative to that of the right [$F(4,45) = 3.03$, $p < 0.03$; Fig. 5.3]. Unilateral injection of saporin into the left VM resulted in a 21% reduction in the relative number of motoneurons compared with that of untreated animals (LSD, $p < 0.02$). Neither exercise nor hormone treatment prevented the saporin-induced reduction in motoneuron number (overall average of 23% reduced; LSDs, $p < 0.001$ compared to untreated animals).

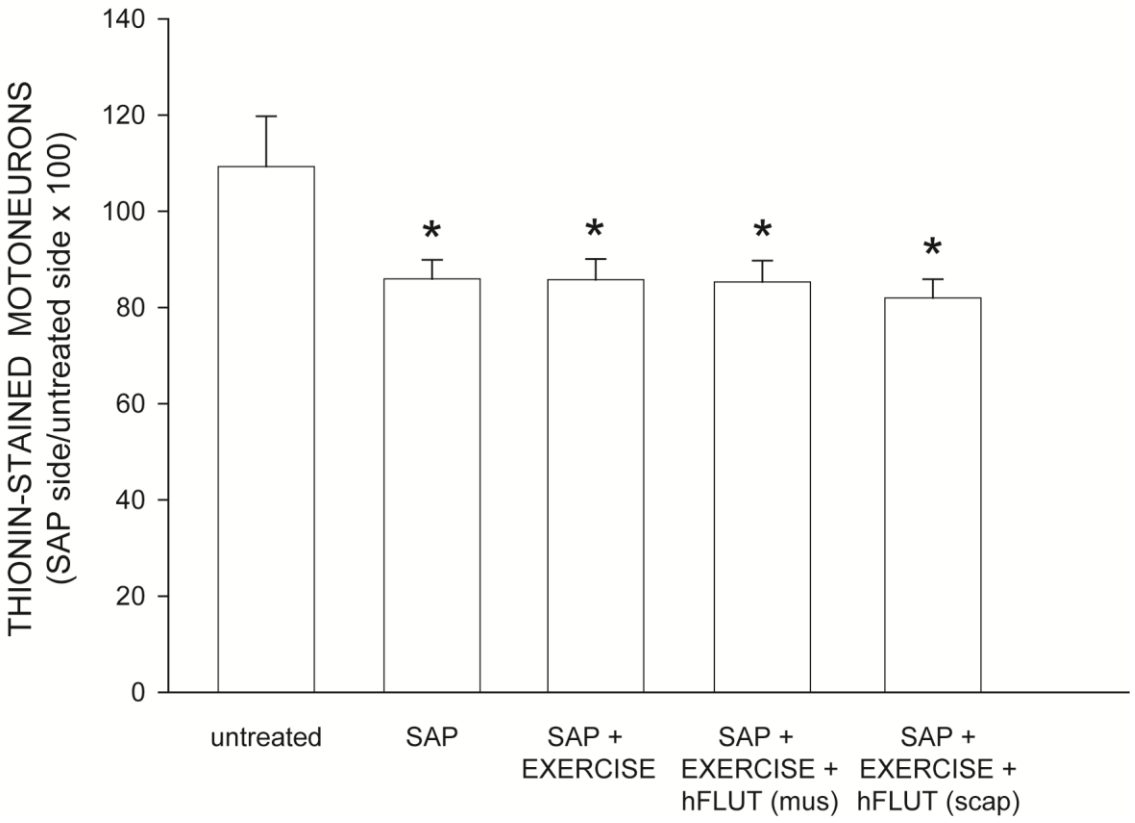


Figure 5.3. Numbers of quadriceps motoneurons in untreated animals and saporin-injected animals that either received no further treatment (SAP), were only given *ad lib* exercise (SAP+EXERCISE), or were given *ad lib* exercise and received a hydroxyflutamide implant at either the vastus lateralis ipsilateral to saporin injection (SAP+EXERCISE + hFLUTmus) or interscapularly (SAP+EXERCISE + hFLUTscap), at four weeks after saporin injection, expressed as a ratio of motoneuron number ipsilateral to the saporin-injected muscle relative to that on the untreated side. Saporin killed approximately 22% of the ipsilateral quadriceps motoneurons, regardless of subsequent treatment. Bar heights represent means ± SEM. * indicates significantly different from untreated animals.

Motoneuron Morphometry

Injection of BHRP successfully labeled quadriceps motoneurons in all groups (Fig. 5.4). The dendritic arbor of labeled quadriceps motoneurons was strictly unilateral, with extensive ramification along the ventrolateral margins of the gray matter and in the lateral funiculus, as well as throughout the ventral horn. An average of 30.33 (± 2.98) motoneurons per animal was labeled with BHRP, and this did not vary across groups [$F(4,31) = 1.98$, *ns*].

Dendritic Length

Surviving quadriceps motoneurons underwent marked dendritic atrophy (Fig. 5.5). Dendritic length was decreased by 64% in saporin-injected animals who received no further treatment compared to that of untreated animals [LSD, $p < .0001$; overall test for the effect of group on dendritic length $F(4,31) = 12.69$, $p < 0.0001$]. Compared to untreated animals, dendritic lengths were significantly shorter in all saporin-injected animals, regardless of exercise status or androgen receptor blockade (LSDs, $p < 0.001$).

Exercise attenuated dendritic atrophy in saporin-injected animals, with dendritic length being reduced on average by only 28%. Saporin-injected animals who exercised had dendritic lengths were 97% longer than those without exercise (LSD, $p < 0.02$).

Androgen receptor blockade at the target muscle prevented the attenuation of dendritic atrophy by exercise in saporin-injected animals. Dendritic lengths in exercised saporin animals with local hydroxyflutamide treatment at the quadriceps were significantly shorter (34%) than those of exercised, but unimplanted, saporin animals (LSD, $p < 0.01$). Furthermore, dendritic lengths in exercised saporin animals with local hydroxyflutamide treatment at the quadriceps were not significantly different from those of saporin animals who received no exercise (LSD, *ns*). Importantly, the attenuation of dendritic atrophy by exercise in saporin-injected animals was not affected by the androgen receptor blockade

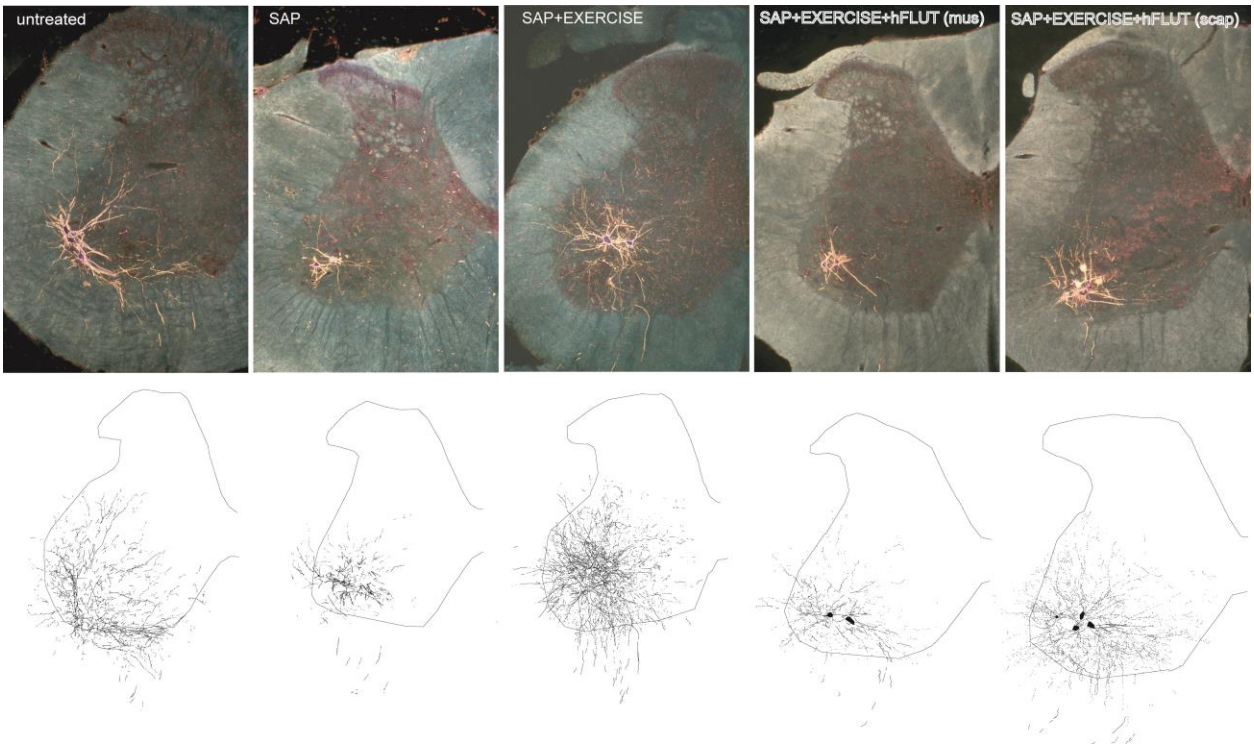


Figure 5.4. Darkfield digital micrographs of transverse hemisections through the lumbar spinal cords and computer-generated reconstructions BHRP-labeled somata and processes of an untreated animal (A,B), and saporin-injected animals with either no further treatment (C,D), given only *ad lib* exercise (E,F), or given *ad lib* exercise in addition to a hydroxyflutamide implant either at the left vastus lateralis ipsilateral to saporin injection (G,H) or placed interscapularly (I,J), after BHRP injection into the left vastus lateralis muscle. Computer-generated composites of BHRP labeling were drawn at 480 μm intervals through the entire rostrocaudal extent of the quadriceps motor pool; these composites were selected because they are representative of their respective group average dendritic lengths. Scale bar = 500 μm.

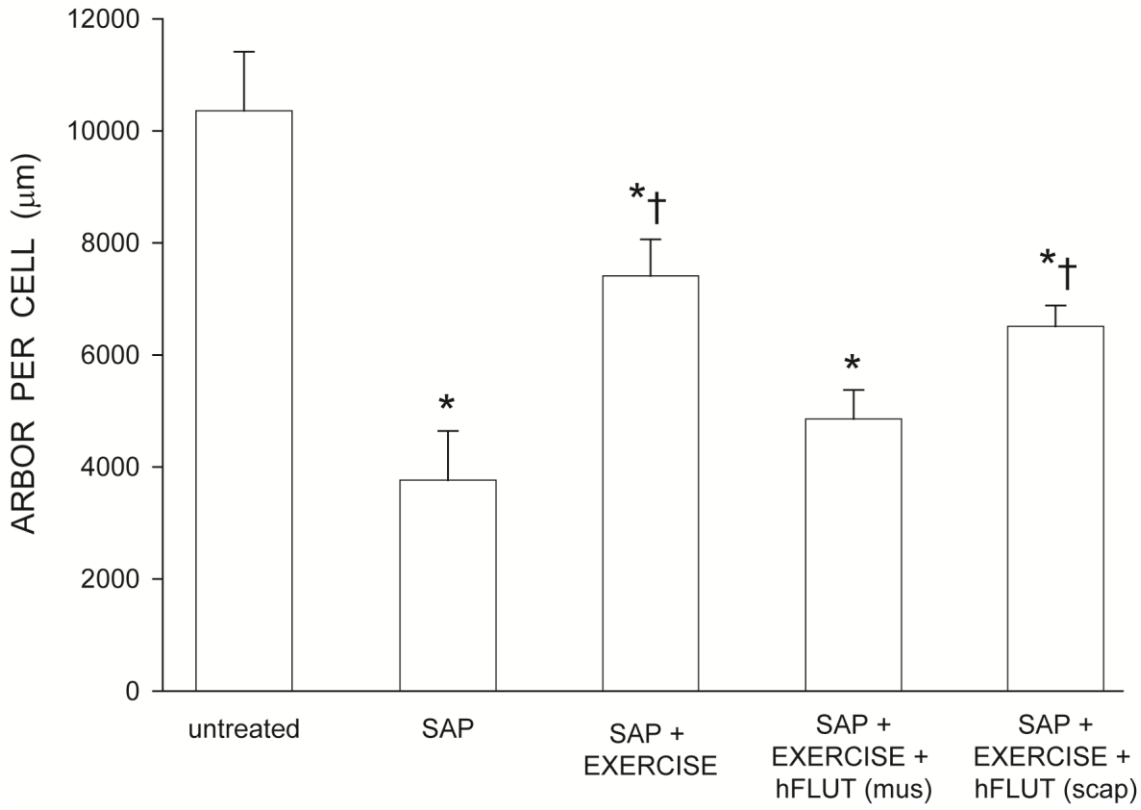


Figure 5.5. Dendritic lengths of quadriceps motoneurons in untreated animals and saporin-injected animals that either received no further treatment (SAP), were only given *ad lib* exercise (SAP+EXERCISE), or were given *ad lib* exercise and received a hydroxyflutamide implant at the vastus lateralis ipsilateral to saporin injection [SAP+EXERCISE + hFLUT(mus)] or interscapularly [SAP+EXERCISE + hFLUT(scap)], at four weeks after saporin injection. Following saporin-induced motoneuron death, surviving neighboring motoneurons lost almost 64% of their dendritic length and exercise attenuated this dendritic atrophy. Androgen receptor blockade at the innervated vastus lateralis prevented the attenuation of atrophy by exercise, while identical implants placed interscapularly did not. Bar heights represent means ± SEM. * indicates significantly different from untreated animals. † indicates significantly different from untreated saporin-injected animals.

delivered interscapularly; dendritic lengths in exercised saporin animals with hydroxyflutamide implants placed interscapularly did not differ from those of exercised, but unimplanted, saporin animals (LSD, *ns*). Furthermore, dendritic lengths in exercised saporin animals with androgen receptor blockade interscapularly had dendritic lengths that were significantly longer (34%) than those of exercised saporin animals with blockade at the quadriceps (LSD, $p < 0.05$).

Dendritic Distribution

Dendritic length was non-uniformly distributed across radial bins, and a repeated measures ANOVA revealed a significant effect of radial location [$F(11,341) = 18.15$, $p < 0.0001$; Fig. 5.6]. Consistent with the results seen in total dendritic length analysis, there was also a significant effect of group [$F(4,341) = 14.59$, $p < 0.0001$]. There were reductions in dendritic length throughout the radial distribution, ranging from 38% (180º to 240º) to 79% (60º to 120º) in saporin-injected animals compared with untreated animals [$F(1,99) = 26.19$, $p < .0007$]. Saporin-injected animals allowed to exercise showed attenuated reductions, with reductions in dendritic length ranging from no change (180º to 300º) to only 54% (60º to 120º) compared to untreated animals [$F(1,99) = 9.44$, $p < .02$]. Throughout most of the radial distribution, dendritic lengths per bin in exercised saporin-injected animals were longer than those of saporin-injected animals who received no further treatment [$F(1,110) = 9.70$, $p < 0.02$], with increases ranging from 61% (180º-240º) to 120% (240º-300º).

Exercised saporin animals who received androgen receptor blockade at the quadriceps showed reductions in dendritic length ranging from 39% (180º to 240º) to 68% (60º to 120º) compared to untreated animals [$F(1,132) = 10.00$, $p < 0.0001$], and these reductions were not significantly different from those seen in saporin animals who received no further treatment [$F(1,143) = .957$, *ns*]. Dendritic lengths per bin in exercised saporin animals who received androgen receptor blockade at the quadriceps

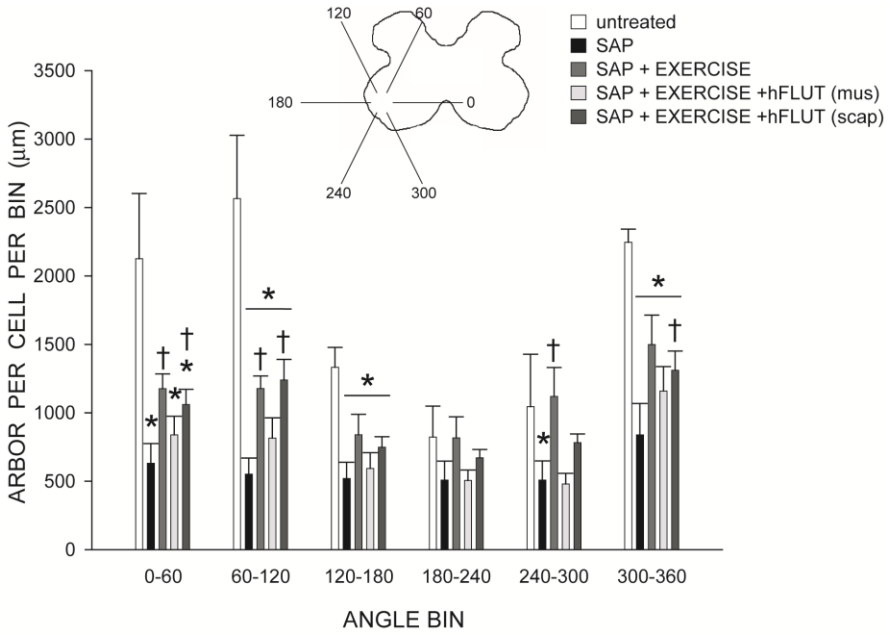


Figure 5.6. Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron dendritic distribution. Length per radial bin of quadriceps dendrites in untreated animals (white bars), and saporin-injected animals that either received no further treatment (SAP, black bars), were given *ad lib* exercise (SAP+EXERCISE, gray bars), or were given *ad lib* exercise and received a hydroxyflutamide implant at the vastus lateralis ipsilateral to saporin injection [SAP+EXERCISE + hFLUT(mus);, light gray bars] or interscapularly [SAP+EXERCISE + hFLUT(scap), dark gray bars]. For graphic purposes, dendritic length measures have been collapsed into 6 bins of 60° each. Quadriceps motoneuron dendritic arbors display a non-uniform distribution, with the majority of the arbor located between 300° and 120°. Following saporin-induced motoneuron death, surviving neighboring motoneurons had reduced dendritic length throughout the radial distribution. Exercise attenuated this reduction, but had no effect in intact animals. Bar heights represent means ± SEM. * indicates significantly different from untreated animals. † indicates significantly different from untreated saporin-injected animals.

were shorter than those of exercised saporin animals who received no androgen receptor blockade [$F(1,143) = 9.11$, $p < 0.01$], with reductions ranging from 23% (300º to 360º) to 57% (240º to 300º).

Exercised saporin animals who received androgen receptor blockade delivered interscapularly showed reductions in dendritic length ranging from 19% (180º to 240º) to 52% (60º to 120º) compared to untreated animals [$F(1,154) = 12.93$, $p < 0.0001$], and these reductions were not significantly different from those seen in saporin animals who received no further treatment [$F(1,143) = 3.61$, *ns*] or exercised saporin animals who received no androgen receptor blockade [$F(1,165) = .053$, *ns*].

Dendritic Extent

In agreement with the nonuniform dendritic distribution of quadriceps motoneurons (Fig. 5.6), radial extent differed across bins (Fig. 5.7), and a repeated measures ANOVA revealed a significant effect of radial bin [$F(11,341) = 26.72$, $p < 0.0001$]. There was also a significant effect of group [$F(4, 341) = 3.23$, $p < 0.03$] due to saporin-induced reductions in dendritic extent; extent was reduced on average 26% across the saporin-treated groups compared to untreated animals. However, unlike dendritic lengths (see above), radial extent did not differ across saporin-treated groups [$F(3,279) = .741$, *ns*].

Rostrocaudal dendritic extent spanned 3776.0 ± 546.3 µm in untreated animals. Neither saporin injection, exercise, nor hydroxyflutamide implantation had an effect; rostrocaudal extent did not differ across groups [overall average 3695.5 ± 125.8 µm; $F(4,31) = .06$, *ns*].

DISCUSSION

Surviving motoneurons respond to the death of neighboring motoneurons with marked dendritic atrophy (Little et al., 2009). Treatment with testosterone, mediated by classical receptor activation, is

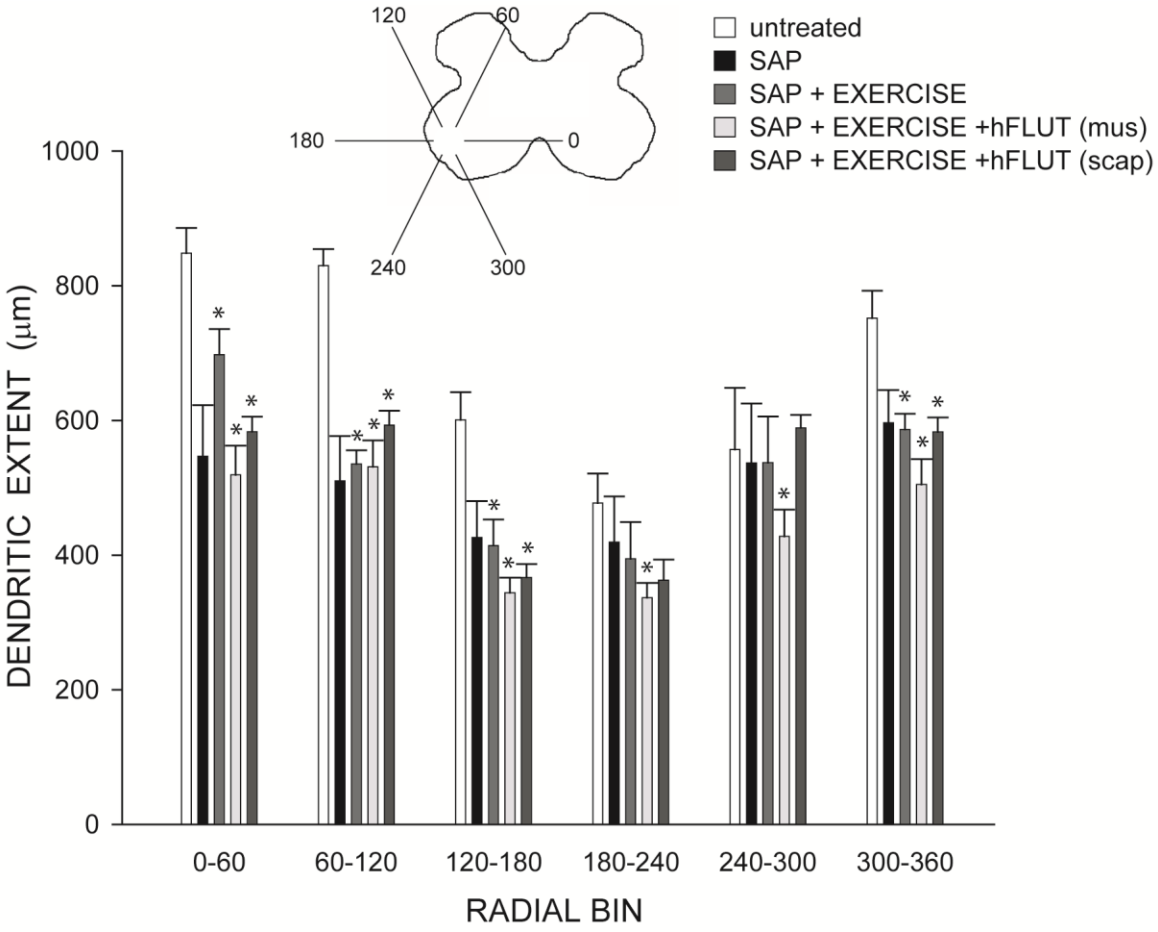


Figure 5.7. Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron radial dendritic extent. Radial extents of quadriceps dendrites in untreated animals (white bars), and saporin-injected animals that either received no further treatment (SAP, black bars), were given *ad lib* exercise (SAP+EXERCISE, gray bars), or were given *ad lib* exercise and received a hydroxyflutamide implant at the vastus lateralis ipsilateral to saporin injection [SAP+EXERCISE + hFLUT(mus);, light gray bars] or interscapularly [SAP+EXERCISE + hFLUT(scap), dark gray bars]. For graphic purposes, dendritic extent measures have been collapsed into 6 bins of 60° each. Bar heights represent means ± SEM. * indicates significantly different from untreated animals.

protective against this atrophy (Cai et al., 2017). Exercise is also protective against dendritic atrophy in surviving motoneurons (Chew and Sengelaub, 2019). In this chapter, I have demonstrated that the neuroprotective effects of exercise are dependent on androgen receptor action specifically at the target muscle.

Running wheel performance

Neither saporin injection nor implantation with hydroxyflutamide impaired the ability of the rats to exercise. Running wheel performance in saporin-treated animals did not differ from that of intact animals, and animals in both groups ran an overall cumulative distance of roughly 124 km over the four weeks of treatment. Similarly, running wheel performance in implanted animals did not differ from that of intact or unimplanted saporin-animals, indicating that differences in dendritic length when determining the muscular site of action were not due to differences in amount of exercise.

Saporin Injection and Muscle Weights

Consistent with the results discussed in previous chapters, saporin injection into the VM reduced muscle weight and the number of innervating motoneurons. This induced death was specific to the motoneurons innervating the saporin-injected VM muscle; there were no changes in the number of BHRP-labeled motoneurons projecting to the adjacent VL. This remains an important consideration for interpreting the effects seen on the morphology of surviving motoneurons. The unchanged number of motoneurons innervating the VL indicates that the changes in their dendritic morphology we observed (see below) cannot be due to accidental spread of saporin in the periphery (and subsequent death of VL motoneurons).

Neither exercise nor hydroxyflutamide treatment prevented saporin-induced decreases in the weight of the injected VM muscle, nor did they prevent saporin-induced motoneuron death. Thus, the

beneficial effects of exercise on the morphology of neighboring surviving motoneurons, and the occlusion of these benefits with androgen receptor blockade at the muscle, cannot be attributed to differences resulting from the degree of peripheral damage or an attenuation of the ability of saporin to kill motoneurons.

The previously noted group difference in the weight of the uninjected (right) in VM animals allowed to exercise, regardless of hydroxyflutamide implantation, was also present in this experiment. As previously mentioned, this group difference was not present in the results discussed in Chapter 3, but was present in the experiment discussed in Chapter 4. The persistence of this hypertrophy in the right VM in exercised groups, and the lack of difference between exercised groups regardless of castration or implantation, lends credence to my hypothesis proposed in Chapter 4. There were still no such group differences observed in either the right or left VL. Thus, it remains unlikely that the protective effects of exercise on dendrites are related to changes in muscle weight.

Specificity of Androgen Receptor Blockade

Androgen receptor blockade at the muscle, but not interscapularly, prevented the protective effects of exercise on dendritic length. Because the amount of hydroxyflutamide in the implants was the same, but interscapular implants were ineffective in preventing the protection of dendritic length by exercise, it cannot be the case that the effects of androgen receptor blockade were the result of systemic exposure. Thus, we can confidently state that the target musculature is the necessary site of androgen action.

Identifying the muscle as the necessary site of action does not inform what cell types or biomolecular mechanisms are responsible for the observed neuroprotective effect. For example, androgens could be acting at muscle fibers, myoblasts, satellite cells, and/or adipose tissue in skeletal muscle, all of which are known to express androgen receptors (Monks et al., 2004; Dubois et al., 2012),

and our current study did not examine which of these cells types could be involved in this neuroprotective mechanism. Monks et al. (2004) noted that androgen receptor expression in myonuclei and fibroblasts was enriched near the neuromuscular junction, and this synaptic enrichment could allow for the androgenic regulation of synapse-specific genes for proteins critical in the maintenance of the innervating motoneurons. An alternative mechanism could incorporate the addition of newly differentiated myoblasts and muscle fibers into the existing structure of skeletal muscle. Androgens have been linked to upregulation of cells differentiating into the myogenic pathway (Hoffman et al., 2009), which could require accompanying neuroplastic accommodations, including remodeling of dendritic structure.

Androgen-Dependent Protection from Dendritic Atrophy

Saporin-induced motoneuron death resulted in a pronounced dendritic atrophy in surviving quadriceps motoneurons (Little et al., 2009). As previously mentioned, this dendritic atrophy is not the result of the loss of afferent fibers from the saporin-injected muscle (Cai et al., 2017) or the increase in activated microglia in the quadriceps motor pool following saporin-induced motoneuron death (Chew et al., 2019). Our lab has proposed that the induced death of motoneurons could result in the release of toxins [e.g., inflammatory cytokines (IL-6, IL-1β, TNF-α), purines (ATP), glutamate, and matrix metalloproteinases (MMPs)] into the extracellular space, that damage the dendrites of surviving motoneurons (Fargo and Sengelaub, 2004; Chew et al., 2019). Such local changes in the cellular microenvironment would be consistent with the general atrophy seen throughout the dendritic distribution rather than a specific atrophy of any given region.

Both exercise and testosterone upregulate antioxidant activity (Powers et al., 1994), the presence of heat shock proteins in skeletal muscle (Salo et al., 1991; Kregel, 2002), and the expression of neurotrophic factors that promote neuroplasticity (e.g., BDNF) and the cytoskeletal protein β-tubulin in

neurons (Jones and Oblinger, 1994). These upregulations are theorized to be adaptive mechanisms in response to the oxidative stress and other biochemical changes experienced during exercise, as androgens have been directly implicated in the positive effects of exercise after neural injury (Wood et al., 2012; Thompson et al., 2014; English et al., 2014) and exercise has commonly been associated with testosterone and its role in anabolic muscle growth (Bhasin et al., 2001, 2003).

The present study provides compelling evidence that the neuroprotective effects of exercise are dependent on androgen action at the target musculature. These results are consistent with similar findings of the importance of gonadal hormones in neuroprotective mechanisms (Kujawa et al., 1989; 1991; Fargo and Sengelaub, 2004a,b; Little et al., 2009; Thompson et al., 2014) and the target musculature as the critical site of action for hormonal effects in the innervating motoneurons, regulating neuron number and dendrogenesis during development, or morphology and protein expression in adulthood (Matsumoto, 1997; Verhovshek et al., 2013; Foecking et al., 2015). For example, transgenic upregulation of androgen receptor expression in somatic musculature confers androgen-sensitivity to the innervating motoneurons (Huguenard et al., 2011). Castration of male rats with enriched androgen receptor in the musculature results in dendritic atrophy in the innervating motoneuron populations that would normally not atrophy following castration, and this atrophy is reversed with androgen replacement. The presence of androgens has also been demonstrated to be necessary for the neurotherapeutic effects of exercise in promoting axonal regeneration following axotomy; castrated rats allowed to exercise show significantly fewer axon sprouts from the distal stump of the regenerating axon compared to gonadally intact rats who undergo the same exercise therapy (Thompson et al., 2014).

Androgen action at the target musculature has also been shown to regulate BDNF expression in motoneurons (Verhovshek et al., 2013) and BDNF is critical in maintaining normal dendritic length in motoneurons (Verhovshek and Sengelaub, 2010). Thus, it is possible that both exercise and

testosterone's neuroprotective effects following partial motoneuron depletion are due to testosterone binding at the target muscle, signaling the muscle and/or the innervating motoneurons to produce trophic factors that protect motoneurons after injury (English et al., 2014; Chew and Sengelaub, 2019). Interestingly, androgen action and BDNF at the target muscle interact to maintain motoneuron dendritic morphology in a manner that varies depending on the androgen sensitivity of the muscle (Verhovshek et al., 2013). This phenomenon is further explored in Chapter 7 of this thesis.

Exercise results in elevations in serum testosterone (Kindermann et al., 1982; Wood et al., 2012; Sato and Iemitsu, 2015), although intensity, duration, prior conditioning, time point of measurement (e.g., immediately vs. hours after exercise; Tremblay et al., 2005; Vingren et al., 2010), or type of training (Tremblay et al., 2004; Sato and Iemitsu, 2015) all contribute to how testosterone concentrations change in response to exercise. Androgens also positively autoregulate expression of their cognate receptor (Mora et al., 1996), suggesting that increases in serum testosterone concentration can also lead to increased expression of androgen receptors. Thus, it is plausible that exercise-driven increases in serum testosterone can drive increased expression of androgen receptors in tissue, including the target muscle. This potential increase in androgen receptor expression at the muscle suggests that exercise may be able to modulate the efficacy of supplemental testosterone treatment compared to sedentary animals, or that exercise may be able to confer resilience to injury if serum androgen concentrations remain elevated. If true, this would demonstrate an effective synergy of both behavioral and hormonal treatments following neural injury.

CONCLUSIONS

Chapter 3 of this thesis has established that exercise is neuroprotective on motoneuron dendrites following the induced death of their neighbors (Chew and Sengelaub, 2019). Here we tested whether the

neuroprotective effect of exercise is dependent on the presence of androgens and, specifically, androgen receptor activation at the target muscle. Our findings indicate that androgen receptor action at the muscle is necessary for exercise-mediated neuroprotection of motoneurons following the death of their neighbors. These findings have identified a necessary site of action and biochemical signaling mechanism for the neuroprotective mechanism underlying exercise treatment, and may provide insight to how the plastic nature of that site of action may be used to improve neurotherapeutic regimens.

CHAPTER 6

DOES EXERCISE UPREGULATE ANDROGEN RECEPTOR EXPRESSION IN SKELETAL MUSCLE?

In prior chapters, I have established that the death of motoneurons has adverse effects on the structure and function of adjacent surviving motoneurons in a rat model of induced motoneuron death, and that either exercise or supplemental testosterone treatment are neuroprotective via androgen receptor activation at the target muscle of the innervating motoneurons (Little et al., 2009; Chung, 2015; Cai et al., 2017; Chew and Sengelaub, 2019). Identification of the receptor dependent mechanism at the target muscle raises several interesting questions regarding whether manipulation of variables pertaining to that mechanism may alter neuroprotective efficacy of hormonal therapy.

The neuroprotective efficacy of supplemental testosterone treatment following partial motoneuron depletion has been studied in two different neural populations, the spinal nucleus of the bulbocavernosus (SNB) and the motoneurons innervating the vastus lateralis (VL). In both populations, induced motoneuron death causes dendrites of surviving motoneurons to atrophy to ~40% of their normal lengths (Fargo and Sengelaub, 2004b; Little et al., 2009). Treatment with testosterone following induced motoneuron death is neuroprotective in both populations, but to differing degrees of effectiveness. In the quadriceps, supplemental testosterone treatment *attenuates* atrophy of surviving motoneuron dendrites; the dendrites are still atrophied compared to normal lengths, but are 50% longer compared to animals who also received CTB-saporin injection but no supplemental testosterone treatment (Little et al., 2009). However, in the SNB, animals who receive the same testosterone treatment show dendritic lengths that are not significantly different from normal animals (Fargo et al.,

2004b). This raises the obvious question of why the same treatment is differentially effective in two different populations.

The SNB and innervated musculature are an androgen-sensitive system (Breedlove and Arnold, 1980, 1983c). Androgens play a critical role in the initial masculinization of the neuromuscular complex during development (Breedlove et al., 1982; Breedlove and Arnold, 1983b), and the complex remains sensitive to androgen manipulation into adulthood (Kurz et al., 1986; Rand and Breedlove, 1995). Specifically, castration of adult male rats causes atrophy of SNB motoneuron dendrites (Kurz et al., 1986) and innervated muscle mass (Wainman and Shipounoff, 1941; Breedlove and Arnold, 1981). This stands in contrast to the more conventional quadriceps motoneurons, in which castration during adulthood does not alter the motoneuron morphology (Huguenard et al., 2011) or muscle weight (Verhovshek et al., 2010). SNB motoneurons accumulate androgens at rates up to three times higher than quadriceps motoneurons (Little et al., 2009), and the SNB-innervated bulbocavernosus and levator ani (BC/LA) muscles express almost four time as many androgen binding sites compared to more conventional skeletal muscles (Dubé et al., 1976). This difference in androgen sensitivity is hypothesized to be the driving force behind the differential neuroprotective magnitude of testosterone treatment following partial motoneuron depletion (Little et al., 2009).

Exercise upregulates serum free testosterone concentrations (Wood et al., 2012), local testosterone metabolism in muscle (Aizawa et al., 2010), and local steroidogenic enzymes in muscle (Aizawa et al., 2011). Testosterone has been linked to many of the physiological adaptations induced following exercise in a dose-dependent manner (Hoffman et al., 2009); these adaptations include increased lean muscle mass, protein synthesis, erythropoiesis, and muscular glycogen storage (Bhasin et al., 2001a; Hoffman et al., 2009). The upregulation of androgen signaling following exercise, either systemically or locally, is likely a means of driving these adaptive changes.

Androgens are known to autoregulate expression of their cognate androgen receptor in a variety of tissues, including skeletal muscle (Antonio et al., 1999; Sinha-Hikim et al., 2004), prostate gland (Prins and Birch, 1993; Mora et al., 1996; Mora and Mahesh, 1999), spinal cord (Freeman et al., 1995), and brain (Lu et al., 1998, 1999). Castration has been found to decrease androgen receptor expression in all of those tissues, while exogenous testosterone replacement normalizes androgen receptor expression (Prins and Birch, 1993; Freeman et al., 1995; Lu et al., 1998, 1999). Therefore, it is likely that an increase in serum testosterone following exercise may also increase androgen receptor expression following exercise.

In Chapters 3-5, I provided evidence that exercise is neuroprotective to motoneuron dendritic atrophy following the death of neighboring motoneurons via androgen receptor action at the target muscle. Here, I have provided evidence as to why I believe a difference in androgen sensitivity of the target muscle is the reason for differential efficacy of testosterone as a neuroprotective treatment in the SNB and quadriceps in the same model of motoneuron death, and that exercise-driven increases in testosterone may cause increased androgen receptor expression in skeletal muscle.

Several prior studies have attempted to quantify whether exercise has an effect on androgen receptor expression in skeletal muscle, using similar motivating rationale that I have outlined above (Deschenes et al., 1994; Matsakas et al., 2004; Willoughby and Taylor., 2004; Ratamess et al., 2005; Spiering et al., 2009; Ahtiainen et al., 2010). These studies have produced conflicting and unclear results on whether exercise influences muscular androgen receptor expression, and differences in exercise paradigms, sampling time point, analytical methods, and model species make interpretation of prior results particularly difficult. Therefore, the aims of the present study are to determine, specifically, whether the *ad lib* exercise paradigm used in prior chapters of this thesis produce the hypothesized increases in serum testosterone concentrations and androgen receptor expression in skeletal muscle.

METHODS AND DESIGN

Animals

Adult male Sprague-Dawley rats (Envigo, Indianapolis, IN) approximately 100 days old were used for this experiment. Rats were single-housed with *ad lib* access to running wheels (width = 11.2 cm; diameter = 37 cm; circumference = 116 cm) attached to their home cages. Exercised rats were allowed to run for four weeks prior to sacrifice (n = 6). In order to determine whether any changes to serum testosterone concentrations or androgen receptor expression persisted following cessation of exercise, some rats were removed from their exercise wheels and placed in new housing without access to exercise wheels following the initial four weeks of exercise (n = 6). A group of untreated and unexercised animals (n = 6) was included. Wheel revolutions were tracked daily to ensure that rats were engaging in exercise throughout the exercise period.

Immnuohistochemical processing

Animals were sacrificed, the thoracic cavity exposed, and blood was collected by cardiac puncture. Following blood collection, the left VL was fresh dissected and flash-frozen in carbon-dioxide cooled methylbutane. Frozen whole muscles were then fixed to a chuck and acclimated at -20° in a cryostat for one hour. Muscles were sectioned transversely at 12µm into three series; two series were sealed in airtight bags and stored in reserve at -80°C, while the remaining series was immediately used for immunohistochemical labeling of androgen receptors and basal laminar membrane.

The immunohistochemical protocol is described in detail in Chapter 2. In summary, sections were incubated for 48 hours in PG-21 anti-androgen receptor antibody (1:1000 dilution; 06-680, Millipore Sigma, Temecula, CA) before a two hour incubation with AlexaFluor 488 fluorescent goat anti-rabbit IgG [1:200; A-11070, Invitrogen, Eugene, OR). Following primary and secondary labeling of

androgen receptors, sections were incubated overnight in D18 anti-laminin primary antibody (1:50; D18, Developmental Studies Hybridoma Bank, Iowa City, IA) prior to incubation in TRITC fluorescent goat anti-mouse IgG (1:200; T-5393, Millipore Sigma, Milwaukee, WI). Slides were coverslipped with Vectashield Hardset mounting medium for Fluorescence (Vector), allowed to dry, the edges sealed with nail polish, and cured overnight at 4ºC. Slides were then stored at 4°C in opaque slide boxes until examination.

Testosterone Assay

Blood samples were allowed to rest at room temperature for one hour after collection to allow for clot formation. The clot was then removed, and samples centrifuged at 4°C for 30 minutes at 2500rpm to separate serum from red blood cells. The sera was then pipetted into fresh tubes and stored at -20°C until assays could be run. Assays for testosterone concentrations were run using a commercial ELISA kit per the manufacturer's recommended specifications (ADI-900-065, Enzo Life Sciences, Farmingdale, NY).

Samples were run in duplicate and diluted 1:40 in the assay buffer provided by the kit manufacturer. All samples were measured within a single assay, and the intra-assay coefficient of variance was 4.35%. Samples with coefficients of variance greater than 12% were excluded. Sample sizes for each group following exclusion are as follows: untreated, n = 6; exercised, n = 4; pre-trained, n = 5.

Microscopy

Androgen receptor-positive puncta were identifiable under FITC HYQ filtered fluorescent illumination (excitation 460-500µm; dichromatic mirror 505µm; barrier 510-560µm), while basal lamina membranes were identifiable under Texas Red HYQ filtered fluorescent illumination (Excitation 532-587µm; dichromatic mirror 595µm; barrier 608-683µm) at x720 final magnification. To ensure that

visualization under each fluorescent wavelength captured the same field of view, the section that was qualitatively determined to have the highest quality of immunolabeling on each slide was first visualized under Texas Red HYQ to confirm that the field of view contained muscle fibers (identified by the presence of basal lamina staining). The fluorescent filters were then rotated while the microscope stage remained unmoved, allowing for FITC HYQ excitation of the same field of view to visualize androgen receptor-positive puncta. Images of basal lamina and androgen receptor-positive puncta staining for each field of view were digitally captured separately in Stereo Investigator. The microscope stage was then moved to view the eight adjacent fields of view surrounding this initial field of view, and images were captured as described above. This process of capturing images for nine fields of view per section was repeated for a single representative section on each slide, and two slides were selected to represent each animal, for a total of 18 fields per animal.

These images were merged in Photoshop 5.5 (Adobe, San Jose, CA) by opening both images, superimposing the images with basal laminar label over that with androgen receptor-positive puncta, and adjusting the opacity of the basal laminar image so that androgen receptor-positive puncta were still visible while being able to identify the muscle fibers ringed by the basal lamina.

Merged images were reimported to Stereo Investigator where the number of muscle fibers and androgen receptor-positive puncta were counted and area of muscle fibers calculated. The total number of androgen receptor-positive puncta from eighteen fields of view of a single animal were summed, and a measure of the density of androgen receptor-positive puncta was calculated by dividing the total number of androgen receptor-positive puncta per animal by the total area of the field of view used to collect all images (each field measured 227852.46μm^2; total area sampled = 4101344.28μm^2), and converting the value to androgen receptor-positive puncta per square millimeter.

Muscle fiber areas were calculated using Stereo Investigator's Nucleator probe (Gundersen et al., 1988). A set of four rays emanating from a point randomly chosen within each muscle fiber was drawn and oriented randomly. Cross sectional areas were measured at a final magnification of 720x were then averaged for each animal for statistical analysis. Area measurements were taken from an average of 295 sampled muscle fibers per animal and produced an average coefficient of error of less than 0.05.

RESULTS

Running Performance

Animals ran consistently over the four weeks they were allowed access to running wheels, averaging 5.02 ± 0.49 (mean ± SEM) kilometers per day (Fig. 6.1). Pre-trained animals were sedentary for an additional four weeks following their exercise period, and thus were not subjected to a different exercise design or experimental manipulation that might affect their running behavior when compared to solely exercised rats [$t(11) =$, *ns*]. Overall, animals ran an average cumulative total of 140.54 ± 13.71 km over the four weeks of *ad lib* exercise.

Serum testosterone concentrations

Plasma testosterone concentrations of untreated rats (11.73 ± 4.00 ng/mL) were within the previously reported range (Heywood, 1980; Stahl et al., 1984). Testosterone levels were increased by almost three times in exercised animals (46.24 ± 22.32 ng/mL), and were significantly elevated over those of untreated animals (LSD, $p < 0.05$). Serum testosterone levels remained elevated four weeks after the exercise period in the pre-trained rats (19.56 ± 4.39 ng/mL) at almost twice that of untreated animals, but this difference failed to reach statistical significance [LSD, p = 0.12; Fig. 6.2; overall $F(2,12) = 2.61$, $p = 0.12$].

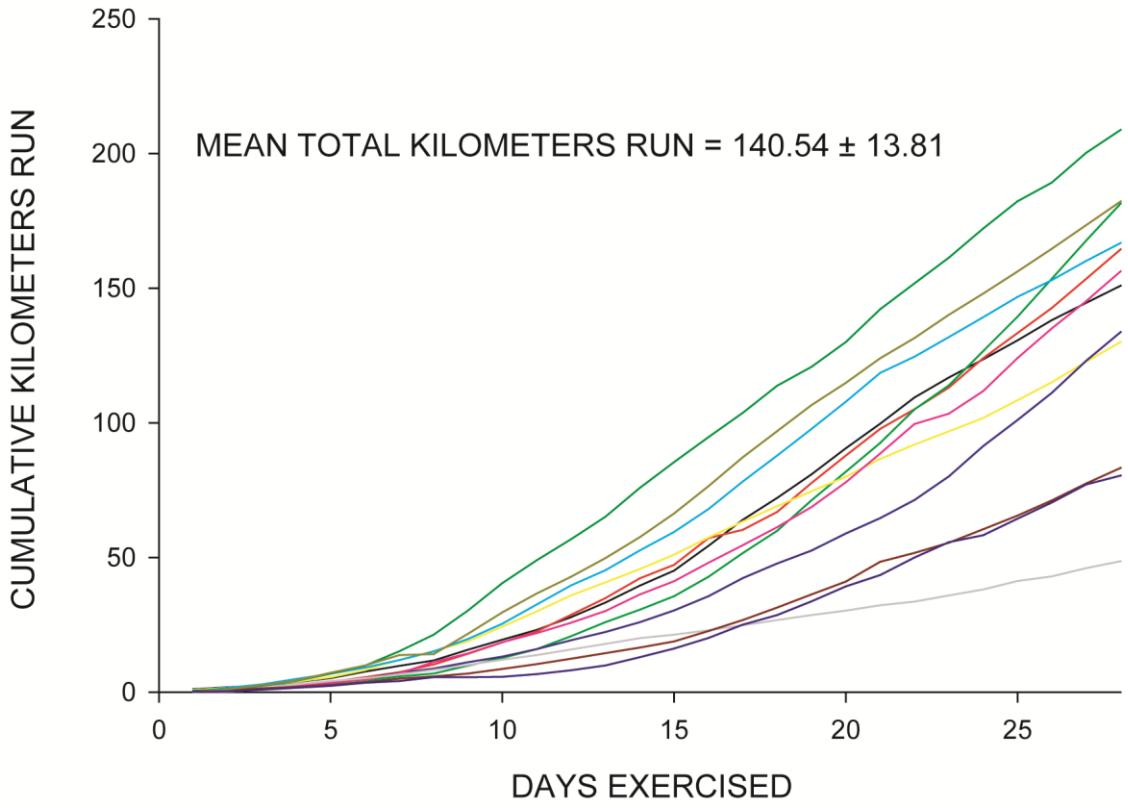


Figure 6.1. Total cumulative distance run in the 28 day exercise period; each line represents the cumulative kilometers run by a single rat. Rats showed a fair amount of variation in both the total cumulative distance run and in the progressive increase in daily distance run. Pre-trained rats were allowed to run for 28 days, and were then placed in housing without access to a running wheel for an additional 28 days. Analysis showed no difference in the distance run by exercised and pre-trained rats.

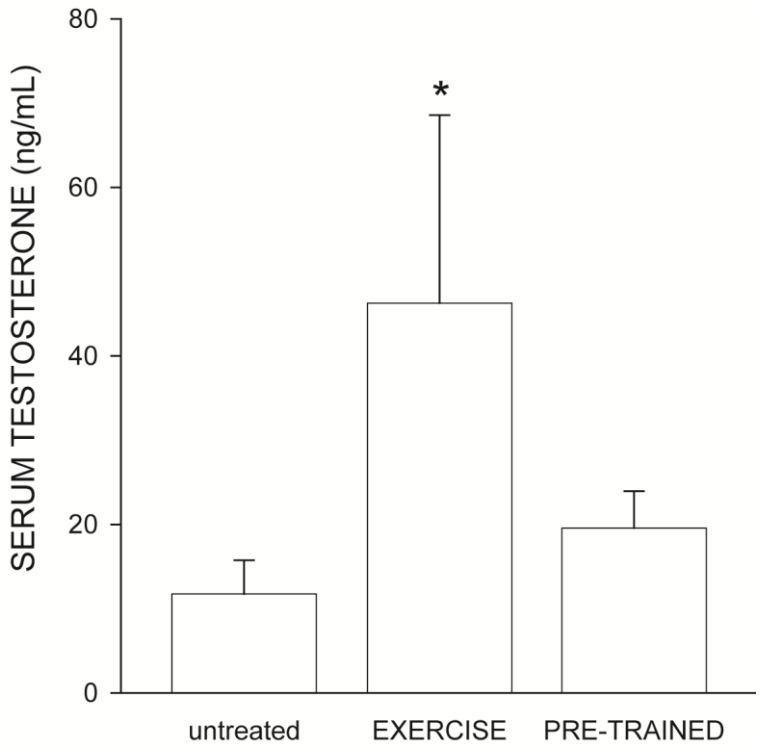


Figure 6.2. Serum concentrations of testosterone in untreated, exercised, and pre-trained rats. Exercised and pre-trained rats were both allowed free-access to running wheels for 28 days (Fig. 6.1), and pre-trained rats were allowed an additional 28 day sedentary period prior to blood collection. Exercised animals showed a large increase in serum testosterone concentrations compared to untreated animals, but with an accompanying large variance in concentrations. Serum testosterone in pre-trained animals was similar to that of untreated animals. Bar heights represent means ± SEM. * indicates significantly different from untreated animals.

Muscle fiber area

There was a significant group effect on VL: muscle fiber cross-sectional area [Fig. 6.3; $F(2,15) = 24.38$, $p < 0.0001$]. Untreated animals had a mean muscle fiber area of 2892 ± 166.04 μm^2. Both exercise and pre-training had an effect on VL muscle fiber area. Cross-sectional area of VL fibers in exercised animals (5636.85 ± 318.12 μm^2) were 95% larger compared to untreated animals (LSD, $p < 0.001$). Muscle fiber area was also increased in pre-trained rats (6957.38 ± 632.65 μm^2) when compared to that of untreated rats (141% increase from untreated; LSD, $p < 0.001$). Muscle fiber areas of exercised and pre-trained rats were also significantly different from each other (LSD, $p < 0.05$).

Androgen receptor expression in skeletal muscle

We were successfully able to immunolabel both androgen receptors and basal laminar membrane in all groups (Fig. 6.4). There was a significant effect of group on androgen receptor-positive puncta density [Fig. 6.5; $F(2,15) = 32.49$, $p < 0.001$]. The average number of androgen receptor-positive puncta/mm^2 in untreated animals was 274.38 ± 33.58. This density of androgen receptor-positive puncta was markedly increased in exercised animals (1550.40 ± 151.62), a 5.7x increase compared to untreated animals (LSD, $p < 0.0001$). The density of androgen receptor-positive puncta was also significantly elevated in pre-trained animals (711.91 ± 121.29) compared to untreated animals (2.6x increase; LSD, $p < 0.02$). The density of androgen receptor-positive puncta in exercised animals was also increased compared to pre-trained animals (LSD, $p < 0.001$).

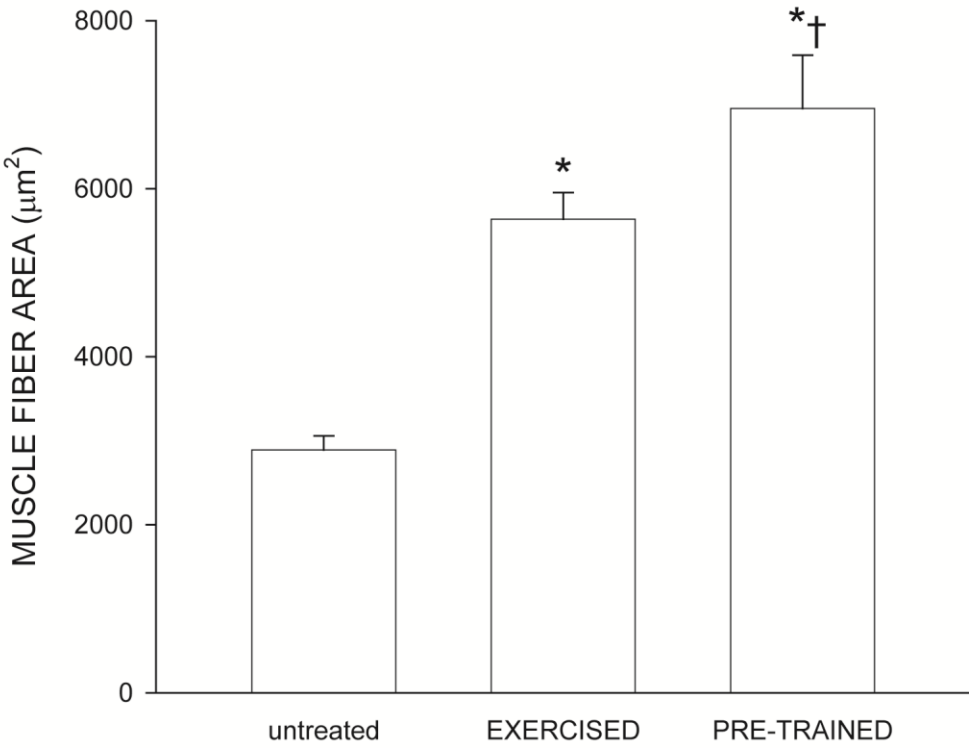


Figure 6.3. Cross sectional area of muscle fibers sampled from animals that were either untreated, allowed to exercise for four weeks (EXERCISED), or allowed to exercise for four weeks followed by a four week sedentary period (PRE-TRAINED). Average muscle fiber cross sectional area was increased in both exercised and pre-trained groups compared to untreated animals, and fiber areas of pre-trained animals were slightly larger than those of exercised animals. Bar heights represent means ± SEM. * indicates significantly different from untreated animals. † indicates significantly different from exercised animals.

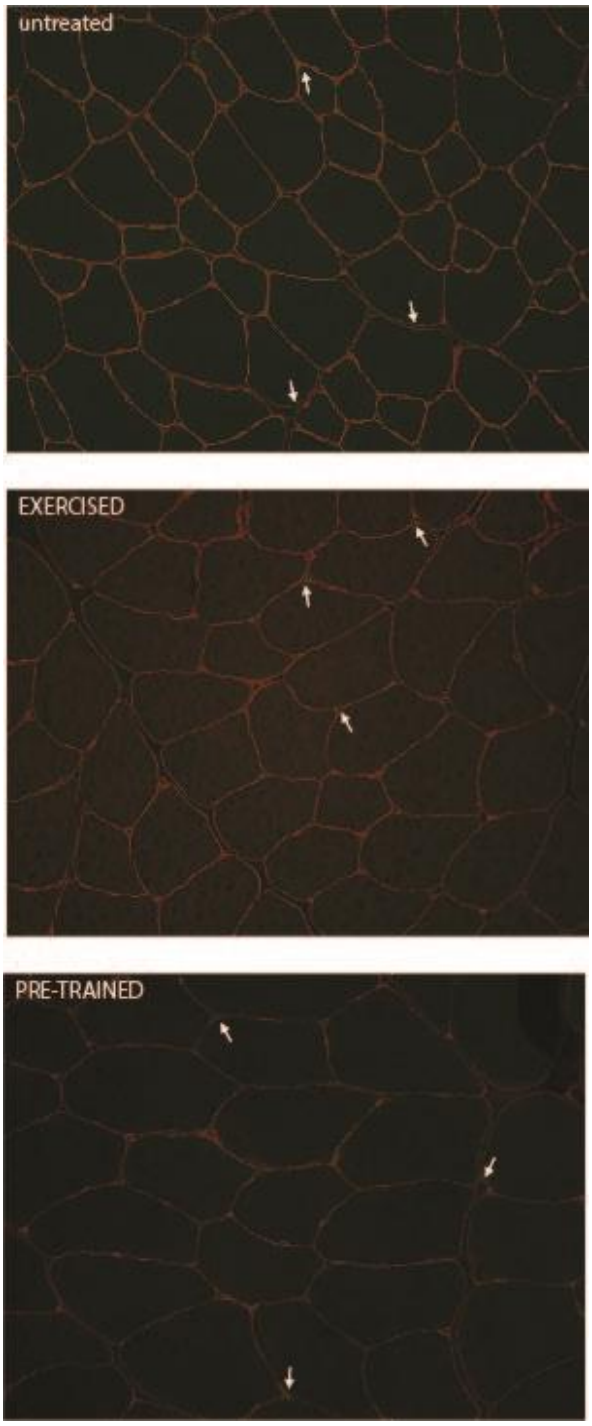


Figure 6.4. Merged image of vastus lateralis muscle tissue fluorescently immunolabeled for both basal laminar membrane (red) and androgen receptor-positive puncta (green) in untreated, exercised, and pre-trained animals. White arrows indicate examples of immnuolabeled androgen receptor-positive puncta.

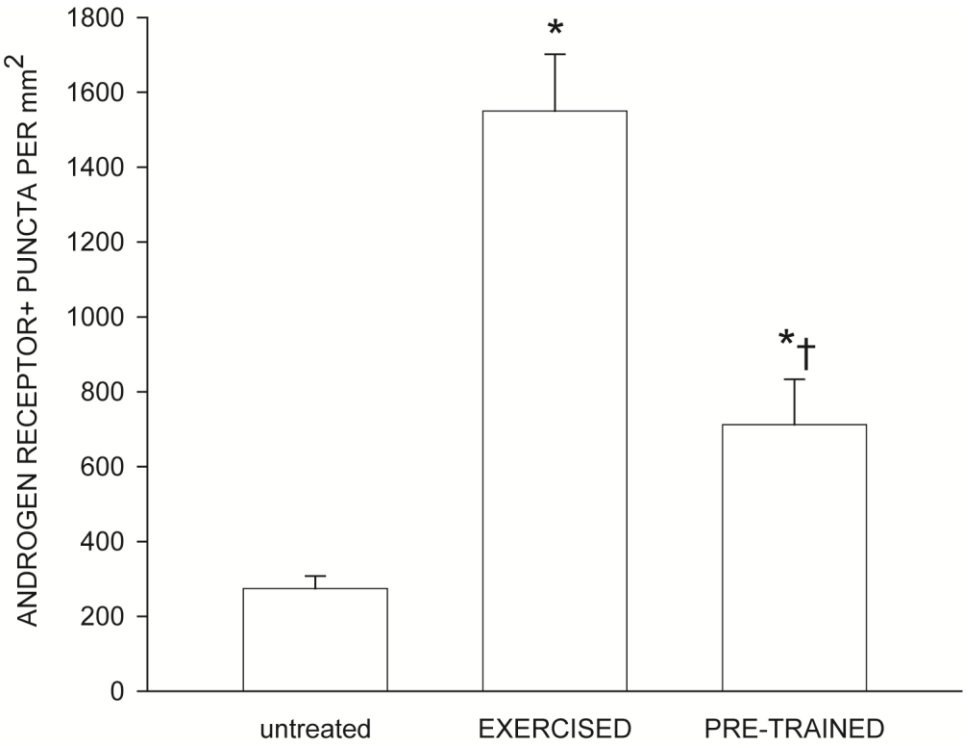


Figure 6.5.Density of androgen receptor-positive puncta in vastus lateralis muscle tissue in animals that were either untreated, allowed to exercise for four weeks (EXERCISED), or allowed to exercise for four weeks followed by a four week sedentary period (PRE-TRAINED). Exercised animals showed an almost six-fold increase in the density of androgen receptor-positive puncta in the tissue compared to untreated animals. Pre-trained animals were also showed an elevation compared to untreated animals, but were significantly reduced compared to exercised animals. Densities are expressed in androgen receptor-positive puncta per square millimeter. Bar heights represent means ± SEM. * indicates significantly different from untreated animals. † indicates significantly different from exercised animals.

DISCUSSION

Exercise effect on serum testosterone

Exercise produced an increase in serum concentrations of testosterone compared to untreated animals. This is consistent with prior reports that have shown that exercise upregulates serum testosterone. Wood et al. (2012) demonstrated that continuous treadmill running, an exercise paradigm very similar to that used in this study, increases rat serum testosterone in a sex-dependent manner. Häkkinen et al. (1988) also found that two years of regular strength training also caused elevations in human basal testosterone, and Tremblay et al. (2004) found that both resistance and endurance exercise were sufficient to cause acute increases in human total testosterone following exercise.

The current data suggests that any increase in basal serum testosterone due to exercise is transient, as pre-trained animals that had exercised for four weeks and were then sedentary for four weeks showed marginally elevated concentrations of testosterone compared to normal animals, but markedly lower concentrations compared to exercised animals. This suggests that maintenance of an exercise regimen is necessary to maintain any elevations in serum testosterone concentrations, and this is mirrored in expression of androgen receptors in skeletal muscle (see below).

In regards to the present study, it is likely that the lack of overall group significance in the elevations of serum testosterone is due to insufficient sample size. I hypothesized that serum testosterone would be upregulated, which would drive upregulation of the androgen receptor at the target muscle due to androgenic autoregulation of the androgen receptor (Mora et al., 1996). Despite the lack of overall group effect on serum testosterone concentrations, there *was* a significant difference in the pairwise comparison of untreated and exercised animals. This significant individual group comparison, large magnitude of the difference (~250% increase in exercised animals), and corroborating

increase in androgen receptor number in the current data seems to indicate that exercise does upregulate muscular androgen receptor expression via upregulation of serum testosterone.

Potential exercise effects on muscle fiber size

Both exercised and pre-trained groups displayed significantly larger cross sectional area of muscle fibers compared to untreated animals. Interestingly, pre-trained animals did show a significant increase in muscle fiber area compared to exercised animals. This additional hypertrophy was unexpected, as hypertrophied muscle fiber areas normally decrease to resemble normal fiber dimensions over the course of 5-15 weeks, depending on fiber composition, following the cessation of exercise (Goldspink and Howells, 1974). Furthermore, pre-trained animals had lower concentrations of serum testosterone compared to exercised animals. This eliminates the possibility that exercise-induced elevation of serum testosterone persists and causes residual hypertrophy at four weeks following the cessation of exercise.

Two possible explanations for the lack of fiber atrophy following the cessation of exercise are the lag of muscle fiber atrophy compared to decreases in serum testosterone after stopping exercise and the age of the rats at the time of sacrifice. The first is fairly straightforward: the four week sedentary period is sufficient to decrease serum testosterone from the exercised phenotype, but is not enough time to observe muscle atrophy. If pre-trained rats were allowed to survive for a longer time period, then it is likely that the additional sedentary time would allow for greater atrophy. This delayed time course of morphological change following androgen deprivation (in this case, *decreased* serum androgen concentrations rather than complete deprivation) has been previously observed. Following castration, serum androgen concentrations drop within hours (Krey and McGinnis, 1990), while corresponding atrophy of BC/LA muscles and innervating SNB somata take place over the course of the following two and four weeks, respectively (Hamson et al., 2009).

Another possible explanation is the age of the rats at time of sacrifice. Rats were all approximately the same age (~100 days old) at the beginning of each experiment, and both pre-trained and exercised rats began exercising for four weeks following arrival. However, pre-trained rats were sedentary for a further four weeks after their four week exercise period. Thus, pre-trained rats were four weeks older than exercised rats at the time of sacrifice. While there are no data to indicate that an additional four weeks of age would impact either serum testosterone concentrations or muscular androgen receptor expression, the additional four weeks did affect body weight; pre-trained rats were qualitatively larger and quantitatively heavier than exercised rats at time of sacrifice. Thus, it is possible that the minor hypertrophy of muscle fiber area in pre-trained rats is due to larger gross size compared to exercised rats.

Exercise upregulates androgen receptor expression in skeletal musculature

Consistent with both serum testosterone concentrations and muscle fiber area, muscular androgen receptors showed a robust group difference. Four weeks of exercise resulted in a substantial increases in the density of VL androgen receptors. Androgen receptor density decreased during the subsequent sedentary period in the pre-trained animals. These data suggest that exercise upregulates muscular androgen receptor expression, and the increase in androgen receptors is transient, reverting towards the density observed in untreated animals as time passes following the cessation of exercise.

Androgen receptors were rare in untreated animals, with an average of only 274 labeled androgen receptor-positive puncta per square millimeter. This is consistent with the findings of Monks et al. (2004), who reported that androgen receptor expression in another skeletal muscle, the extensor digitorum longus (EDL), is relatively rare. Only 7% of myonuclei in the EDL were positive for androgen receptor label, compared to 74% in levator ani myonuclei (LA; Monks et al., 2004). Qualitative comparison of androgen receptor expression in the BC/LA musculature [used in the

development of our immunofluorescent androgen receptor protocol; Figs. 2.4, 2.6] and the VL also showed a difference similar to that noted by Monks et al. (2004). This extremely large difference in the prevalence of androgen receptors is representative of the difference in androgen receptor expression in more typical somatic (EDL) and androgen-sensitive (LA) skeletal musculature.

Exercise caused a substantial upregulation in the density of androgen receptors in the VL. Compared to untreated animals, exercised animals had almost six times as many androgen receptors per unit area. While direct comparisons to Monks et al. (2004) cannot be made due to differences in the method of androgen receptor quantification [i.e., percent of androgen receptor-positive myonuclei in Monks et al. (2004) vs. number of androgen receptor-positive puncta/mm^2], it is interesting to note that the LA expressed roughly 10x as many androgen receptor positive myonuclei compared to the EDL. This relative difference in androgen receptor expression between an androgen-sensitive and more typical somatic muscle begs the question of whether exercise may confer androgen-sensitivity in muscles that normally are not androgen sensitive (addressed in Chapter 8).

Pre-trained animals showed an intermediary phenomenon when compared to both untreated and exercised animals. To reiterate, pre-trained animals were allowed to exercise for the same four week period as the exercised animals, but were then placed in new housing sans running wheel for an additional four weeks; the length of this second four week period was chosen because the recovery period following saporin injection was also four weeks. This sedentary period allowed us to determine whether any changes to androgen receptor expression seen after exercise would persist through the recovery post-saporin. Although the density of androgen receptors was still elevated in pre-trained animals that had four weeks of rest over that of untreated animals, androgen receptor density was also significantly decreased compared to exercised animals. The persistence of the increase in androgen receptors compared to untrained animals indicates that my hypothesis of exercise conferring resilience

to subsequent neural injury is still plausible, although it remains unclear whether there is a threshold effect that governs how many additional androgen receptors are necessary to confer any hypothesized resilience.

Localization of the androgen receptor in skeletal muscle

In all observed groups, androgen receptor expression was localized to isolated pockets within the basal lamina but predominantly in areas outside of the basal lamina, between muscle fibers. This was in opposition to our initial hypothesis of where the androgen receptors would be located. We had noted that many androgen receptors were located within the muscle fiber during our pilot trials of the immunohistochemical protocol with the androgen-sensitive bulbocavernosus and levator ani muscle (Figs. 2.4-2.6). Because the goal of the present study was to quantify gross expression of androgen receptor within the gross structure of skeletal muscle – the necessary site of androgen action for exercise-driven neuroprotection identified in Chapter 5 – rather than the expression specifically within muscle fibers, the measure of androgen receptor density per square millimeter was used rather than the initially planned measure of androgen receptor per muscle fiber.

Although we did not co-label with DAPI or other means of labeling cell nuclei, anatomical similarities in the structure and location of these laminar pockets in the tissue collected for this study and reports from prior studies lead me to believe that these pockets within the basal lamina were representative of myonuclei, which have previously been reported to be sites of androgen receptor reactivity (Monks et al., 2004; Sinha-Hikim et al., 2004). In addition, the location of androgen receptors in the inter-fiber areas of my tissue could indicate the presence of androgen receptor label in satellite cells or myoblasts, which are thought to be the predominant location of androgen receptor expression in skeletal muscle (Sinha-Hikim et al., 2004; Chen et al., 2005). Although such statements must be limited to speculation, as the collected tissue was not co-labeled for markers specific to satellite cells or

myoblasts. However, identification of the specific cell types within the muscle that androgen receptors are localized to was not a primary goal of the present study. The experiment discussed in Chapter 5 had identified the target muscle as the necessary site of androgen action for exercise-driven neuroprotection, and the current study was designed only to determine whether exercise upregulates androgen receptor expression within the gross structure of the muscle.

Despite the present inability to confirm the cell types where androgen receptors were present, the current literature does offer some support for my interpretation that androgen receptor label in the present study partially localized to satellite cells. Satellite cells are quiescent precursor cells found within the gross structure of the muscle, but are located outside of the sarcolemma and basal lamina encasing individual muscle fibers (Hawke and Garry, 2001). Following stress or myotrauma (e.g., exercise), satellite cells begin a process of proliferation and differentiation into the myogenic lineage, and are reclassified as myoblasts (Seale and Rudnicki, 2000). Eventually, myoblasts are integrated into existing muscle fibers or multiple myoblasts fuse to form new muscle fibers as a means of repairing muscle tissue (Seale and Rudnicki, 2000; Hawke and Garry, 2001). Importantly, this process is mediated by androgens. Satellite cells express androgen receptors (Sinha-Hikim et al., 2004), and administration of androgens is correlated with an increased number of satellite cells, androgen receptor expression, and changes to satellite cell ultrastructure that is indicative of proliferation (Joubert and Tobin, 1995; Sinha-Hikim et al., 2003, 2004).

Together, this provides strong evidence for the following model: exercise induces muscular damage and upregulates testosterone production, androgens then signal to satellite cells within the muscle, and satellite cells enter the myogenic lineage to repair and strengthen the muscle. This model would also account for the decrease in muscular androgen receptor expression in pre-trained animals when compared to exercised animals in the present study. Pre-trained animals would not be damaging

their muscles during their four week sedentary period, and thus not require as many activated satellite cells to repair muscle damage. Because satellite cells are one of the primary locations of androgen receptors in muscle tissue (Sinha-Hikim et al., 2004), this hypothesized decreased number of satellite cells could account for the fewer number of androgen receptors observed in the pre-trained group from the present study.

Implications of androgen receptor upregulation

As previously stated, the current data show that exercise upregulates the expression of androgen receptors in skeletal musculature. The experiments in Chapters 4 and 5 have established that exercise is neuroprotective in a manner that is androgen dependent and, specifically, requires androgen receptor activation at the target muscle. Taken together, this modulation of androgen receptor expression at the known site of action raises the question of whether changing androgen sensitivity at such a critical site can modulate the effectiveness of subsequent androgen treatment.

It has been previously established that treatment with testosterone can be neuroprotective against motoneuron dendritic atrophy in a receptor dependent manner (Little et al., 2009; Cai et al., 2017), and that the same dosage of testosterone is neuroprotective to a greater degree in an androgen-sensitive neuromuscular system (Fargo et al., 2004a,b). This leads me to speculate that animals who have exercised prior to receiving a neural injury may show an increased effectiveness of testosterone treatment in attenuating dendritic atrophy compared to sedentary animals who receive the same testosterone treatment due to the already heightened androgen sensitivity of the necessary target muscle in exercised animals.

CONCLUSIONS

The results from the present experiment indicate that exercise upregulates androgen receptor expression in skeletal muscle, and that cessation of exercise causes receptor expression to revert towards normal levels as time passes after exercise has stopped. Exercise also has a significant effect on serum testosterone concentrations and causes an increase in muscle fiber size. This modulation of androgen receptor expression at the known site of androgen-dependent neuroprotective action suggests that exercise prior to receiving an injury may alter the efficacy of testosterone treatment.

CHAPTER 7

DOES EXERCISE PRIOR TO NEURAL INJURY REDUCE THE SEVERITY OF MOTONEURON DENDRITIC ATROPHY FOLLOWING THE DEATH OF THEIR NEIGHBORS?

We have established that the loss of motoneurons has adverse effects on the structure and function of surviving motoneurons using a rat model of motoneuron death, including dendritic atrophy and a resulting decrease in electrophysiological excitability (Little et al., 2009; Cai et al., 2017). This induced atrophy and decrease in excitability is responsible for at least some of the movement deficits that accompany disease or injury-related loss of motoneurons. Given that we currently lack the technology to replace dead motoneurons, protecting surviving motoneurons from injury-induced atrophy is an important goal.

We have also shown that *ad lib* exercise is neuroprotective against dendritic atrophy, to a degree comparable to that seen when rats are treated with testosterone (Chew and Sengelaub, 2019). In Chapters 4 and 5 of this dissertation, I have demonstrated that exercise is neuroprotective through an androgen-dependent mechanism, specifically requiring androgen receptor activation at the target muscle, the vastus lateralis (VL) muscle of the quadriceps.

Treatment with exogenous testosterone is effective in attenuating motoneuron dendritic atrophy in both the androgen-sensitive spinal nucleus of the bulbocavernosus (SNB; Fargo and Sengelaub, 2004a,b) and more typically somatic quadriceps motoneuron populations (Little et al., 2009). Interestingly, the same dosage of testosterone treatment attenuates dendritic atrophy to different degrees in these two populations. In the SNB, dendrites in testosterone-treated animals following partial

motoneuron depletion are comparable to normal lengths (Fargo & Sengelaub, 2004b). However, in the quadriceps, VL dendrites of testosterone-treated animals following partial motoneuron depletion are ~60% of normal length (Little et al., 2009). This begs the question of why the same dosage of testosterone is neuroprotective to different degrees in these two populations.

Motoneurons and their innervated targets have been shown to have coregulatory effects on each other, and both exercise (Chapter 5) and testosterone treatment (Chung, 2015) in the quadriceps have been demonstrated to be neuroprotective via steroid receptor action at the target muscle. Furthermore, the bulbocavernosus and levator ani musculature (BC/LA) innervated by the SNB express androgen receptors in quantities up to seven times higher than observed in somatic skeletal muscle, such as the quadriceps (Krieg, 1976). This increased androgen sensitivity of the BC/LA, combined with the known mechanism requiring steroid receptor activation at the target muscle, provides strong supporting evidence that the difference in the magnitude of neuroprotection of the same testosterone treatment in the SNB and quadriceps could be due to the difference in the number of androgen receptors at the respective target muscles.

Androgen receptors in the target muscle can have important modulatory effects on motoneuron properties. Normally, castration of adult male rats produces dendritic retraction in the motoneurons of the SNB (Kurz et al., 1986), but not those of the quadriceps (Huguenard et al., 2011). This difference in motoneuron morphology following castration is attributed to the highly androgen-sensitive nature of the SNB (Huguenard et al., 2011). Transgenic upregulation of the number of androgen receptors expressed in the quadriceps via knock-in of the human skeletal actin promoter (HSA-AR Tg) can confer androgen sensitivity to quadriceps motoneurons; castration of these transgenic rats produces dendritic atrophy in quadriceps motoneurons, which normally do not atrophy following castration (Huguenard et al., 2011). While the induced sensitivity of quadriceps motoneurons to castration is not direct evidence of increased

sensitivity to testosterone treatment, it does demonstrate that modulation of androgen receptors in the target muscle can alter the innervating motoneuron properties.

In Chapter 6, I demonstrated that exercise upregulates androgen receptor expression in the VL and that serum testosterone is elevated after four weeks of exercise. If my proposed model in which androgen receptor sensitivity governs the efficacy of testosterone treatment is valid, then this exercise-induced upregulation of androgen receptors in the target muscle may increase the efficacy of supplemental testosterone in protecting motoneuron dendrites from atrophy following the death of adjacent motoneurons. Furthermore, it is also possible that the increased androgen sensitivity may attenuate the magnitude of dendritic atrophy without any testosterone treatment, due to the increase in circulating testosterone following exercise. Therefore, the aims of the current experiment are to determine whether exercise prior to neural injury 1) confers resilience to dendritic atrophy in the absence of subsequent treatment, and 2) alters the degree to which testosterone protects dendrites from atrophy.

METHODS AND DESIGN

Animals

Adult male Sprague-Dawley rats (Envigo, Indianapolis, IN) approximately 100 days old were used for this experiment. In order to assess whether prior exercise affects dendritic atrophy following subsequent partial motoneuron depletion, some rats were pre-trained prior to any other manipulations. Pre-trained rats were allowed free access to exercise wheels (width = 11.2 cm; diameter = 37 cm; circumference = 116 cm) attached to their home cages for four weeks (n = 4). Following the pre-training period, some rats were subjected to the partial motoneuron depletion injury model used in Chapters 2-5.

In brief, we used the toxin saporin, conjugated to the cholera toxin B subunit (CTB-saporin), to kill motoneurons. Rats were anesthetized with isoflurane, and the left vastus medialis (VM) muscle was exposed and injected with CTB-saporin (2 µL, 0.1%; Advanced Targeting Systems, Inc., San Diego, CA). Some rats were not treated further (n = 4), whereas others simultaneously had 45mm Silastic implant filled with crystalline testosterone placed interscapularly (n = 4; 4-androsten-17β-ol-3-one; Steraloids, Newport, RI).

In order to compare whether pre-training was neuroprotective, additional groups of rats that were **not** pre-trained were also injected with CTB-saporin at the left VM. Some rats were not treated further (n = 6), whereas others simultaneously received a Silastic implant filled with crystalline testosterone (n = 6).

Wheel revolutions were tracked daily to ensure that rats were engaging in exercise throughout the recovery period. A group of untreated and unexercised animals (n = 5) was included. Because some of the animals in the study were not included in all analyses due to histological or histochemical compromise, group sizes for each analysis are reported individually below (overall n = 29).

Histochemical and Histological Processing

Four weeks after saporin injection, animals were reanesthetized, and the left VL muscle was exposed and injected with BHRP (2 µL, 0.2%; Invitrogen, Carlsbad, CA), as described in Chapter 2. Animals were sacrificed, exsanguinated, and fixed (1% paraformaldehyde/1.25% glutaraldehyde). To confirm the specificity of the saporin injections, the VM and VL were dissected bilaterally and weighed, and the lumbar spinal cords were removed and prepared for histochemical processing. Spinal cords were post-fixed and cyroprotected overnight, then embedded in gelatin, frozen, and sectioned transversely at 40 µm into four alternate series. One series was stained with thionin for use in cell counts, and the remaining three series were immediately reacted to visualize BHRP using the tetramethyl benzidine

protocol described in Chapter 2. Once reacted, sections were mounted on gelatin-coated slides, and counterstained with thionin.

Microscopy

Motoneuron Counts

Motoneuron counts were collected in order to confirm that CTB-saporin injection was effective in inducing motoneuron death. The method is more extensively detailed in Chapter 2. In summary, thionin-stained motoneurons in the left and right lateral motor columns were stereologically counted along the rostrocaudal distribution of BHRP-labeled motoneurons. Raw counts were corrected for sampling, and a ratio of the number of motoneurons in the left and right lateral motor columns was calculated in order to determine whether there were fewer motoneurons on the CTB-saporin injected left side when compared to the uninjected right side. This has proven to be a reliable indicator of assessing whether CTB-saporin successfully induced the death of motoneurons (Little et al., 2009; Cai et al., 2017; Chew et al., 2019).

Motoneuron counts were derived from a mean of 10.46 sections spaced 480 µm apart and distributed uniformly through the rostrocaudal extent of the quadriceps motoneuron pool range. This sampling scheme produced an average estimated coefficient of error (CE) of .059. (untreated, n = 5; SAP, n = 6; SAP + T = 6; PRE-TRAINED, n = 4; PRE-TRAINED + SAP, n = 4; PRE-TRAINED + SAP + T, n = 4).

Using similar methods, the number of BHRP-labeled motoneurons was assessed in all sections of the reacted series through the entire rostrocaudal extent of their distribution for all animals. Counts of BHRP-labeled quadriceps motoneurons were made under brightfield illumination, where somata could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed (untreated, n = 5; SAP, n

= 6; SAP + T = 6; PRE-TRAINED, n = 4; PRE-TRAINED + SAP, n = 4; PRE-TRAINED + SAP + T, n = 4).

Motoneuron Morphometry

Measures of motoneuron morphometry were collected from one of the three series that underwent histochemical processing to visualize BHRP label. Motoneurons labeled with BHRP were counted in a similar fashion described above for cell counts in the non-histochemically reacted series. Measures of dendritic morphometry were collected by reconstructing BHRP-labeled dendritic arbors from individual sections and merging all reconstructed sections from a single animal into a single composite reconstruction. This composite was used to find the summed total length of labeled dendrites and analyze the radial distribution, radial extent, and rostrocaudal extent of dendritic label. These methods and their rationale are described in detail in Chapter 2 (untreated, n = 5; SAP, n = 6; SAP + T = 6; PRE-TRAINED, n = 4; PRE-TRAINED + SAP, n = 4; PRE-TRAINED + SAP + T, n = 4).

RESULTS

Running Performance

Animals ran consistently over the four weeks they were allowed access to running wheels, averaging 5.45 ± 0.17 (mean ± SEM) kilometers per day (Fig. 7.1). Analysis revealed that there were no group differences in total cumulative distance run [$F(2,9) = 2.49$, *ns*]. Thus, all groups were considered to be similarly trained prior to receiving saporin or testosterone treatment. Overall, animals ran an average cumulative total of 152.50 ± 15.00 km over the four weeks of *ad lib* exercise.

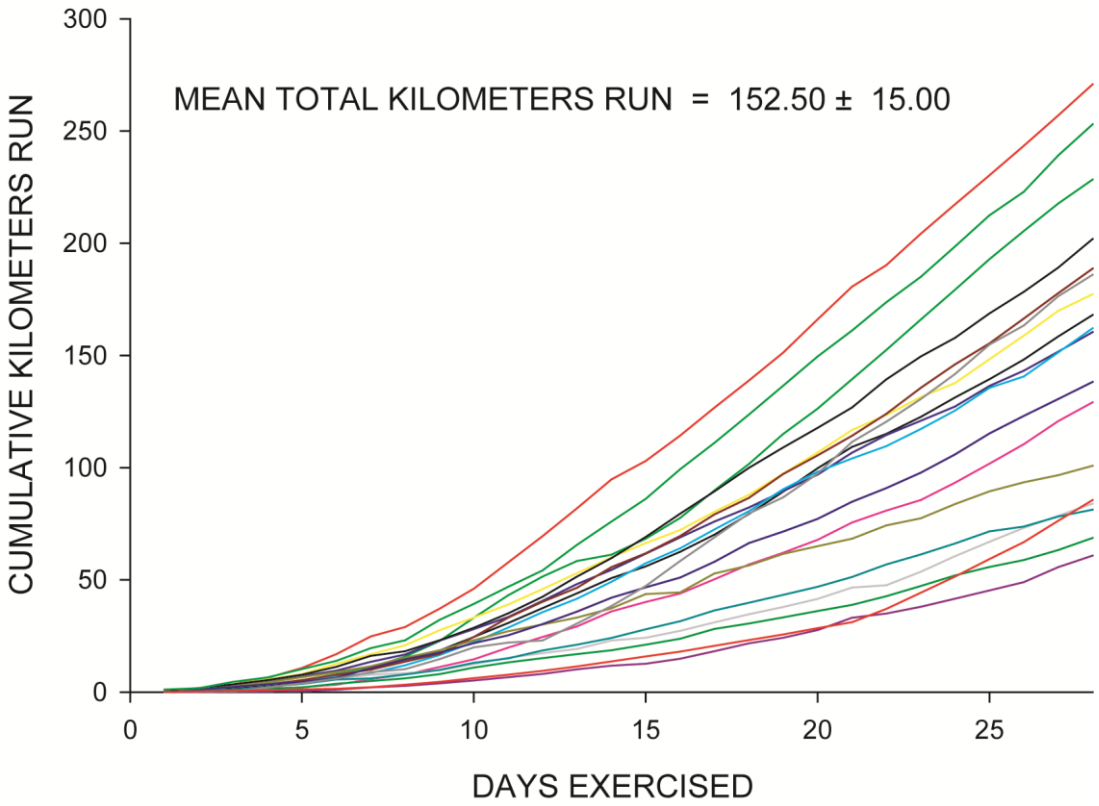
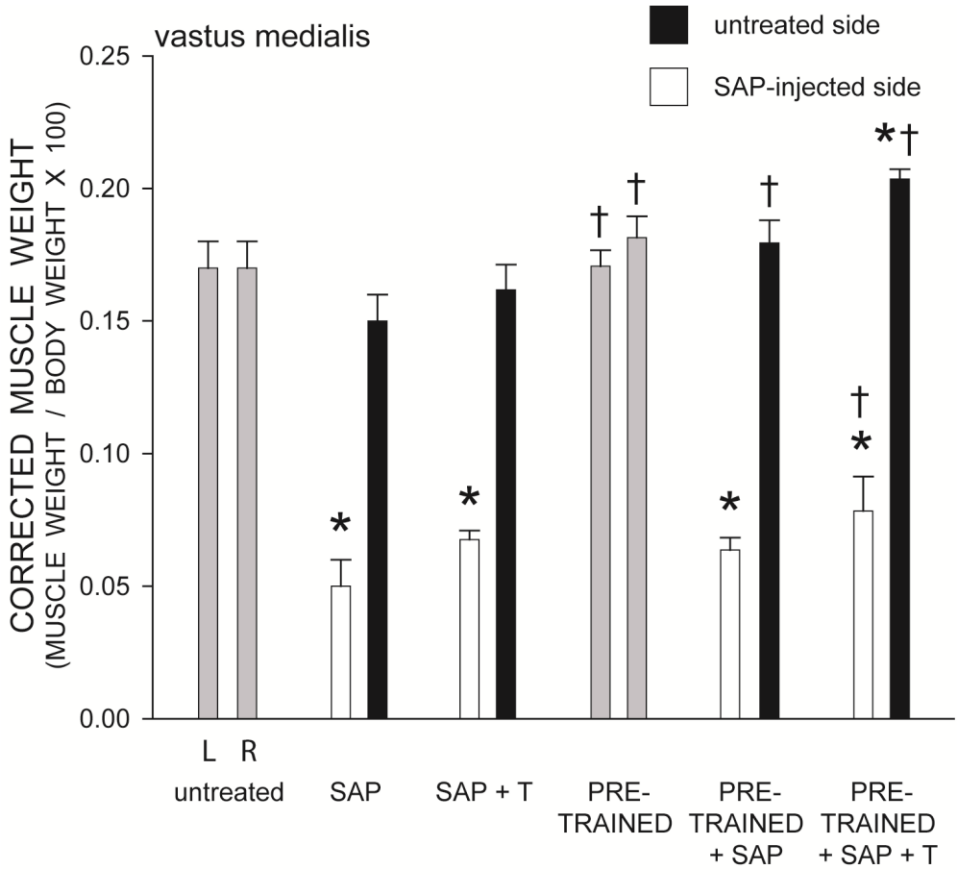


Figure 7.1. Total cumulative distance run in the 28 days prior to injection of saporin; each line represents the cumulative kilometers run by a single rat. Rats showed a fair amount of variation in both the total cumulative distance run and in the progressive increase in daily distance run. Rats were allowed to run prior to other experimetnal manipulations, but retrospective analysis revealed that there were no group differences in either total cumulative distance nor daily distance run by rats across groups.

Muscle Weights

Group difference in body weight were present [$F(5,27) = 3.44$, $p < 0.02$], and thus raw muscle weights were corrected for body mass to assess potential effects of saporin, hormone treatment, and/or exercise on muscle weight (Fig. 7.2). In untreated animals, the corrected weights of the right (0.17 ± 0.01) and left (0.17 ± 0.01) VM muscles were similar [$t(4) = 2.14$, *ns*]. There was a significant effect of group on the weights of the uninjected (right) VM muscle [$F(5,27) = 4.07$, $p < 0.001$]. Pre-trained animals had larger corrected right VM muscle weights (0.19 ± 0.01) than animals not allowed to exercise (0.16 ± 0.01; average increase of 19%). All pre-trained animals, regardless of hormonal treatment or saporin injection, had significantly larger right VM weights than untrained animals who received saporin and no testosterone treatment (LSDs, $p < 0.05$). Pre-trained animals that received saporin and testosterone treatment had significantly larger right VM weights compared to all untrained animals, regardless of saporin injection or hormone treatment (LSDs, $p < 0.05$). Uninjected VM weights across pre-trained groups did not differ from each other [LSDs, *ns*].

Injection of saporin into the left VM resulted in muscle atrophy in the saporin groups [overall average of 68% reduction in weight; $F(5,27) = 51.49$, $p < 0.0001$]. Compared to those of untreated animals, saporin-injected animals that received no further treatment had VM weights that were 74% lighter (LSD, $p < 0.0001$). Pre-training did not prevent subsequent saporin-induced weight loss in the left VM; compared to those of untreated animals, pre-trained saporin-injected rats had VM weights that were 63% lighter (LSD, $p < 0.0001$). Pre-trained animals who received saporin and testosterone treatment had significantly larger left VM weights compared to untrained animals who received saporin and no hormone treatment (LSD, $p < 0.01$), but muscle weights across all other saporin groups did not differ from each other [LSDs, *ns*].


vastus medialis
untreated side
SAP-injected side
CORRECTED MUSCLE WEIGHT
(MUSCLE WEIGHT / BODY WEIGHT X 100)
0.25
0.20
0.15
0.10
0.05
0.00
L R
untreated
SAP
SAP + T
PRE-TRAINED
PRE-TRAINED + SAP
PRE-TRAINED + SAP + T

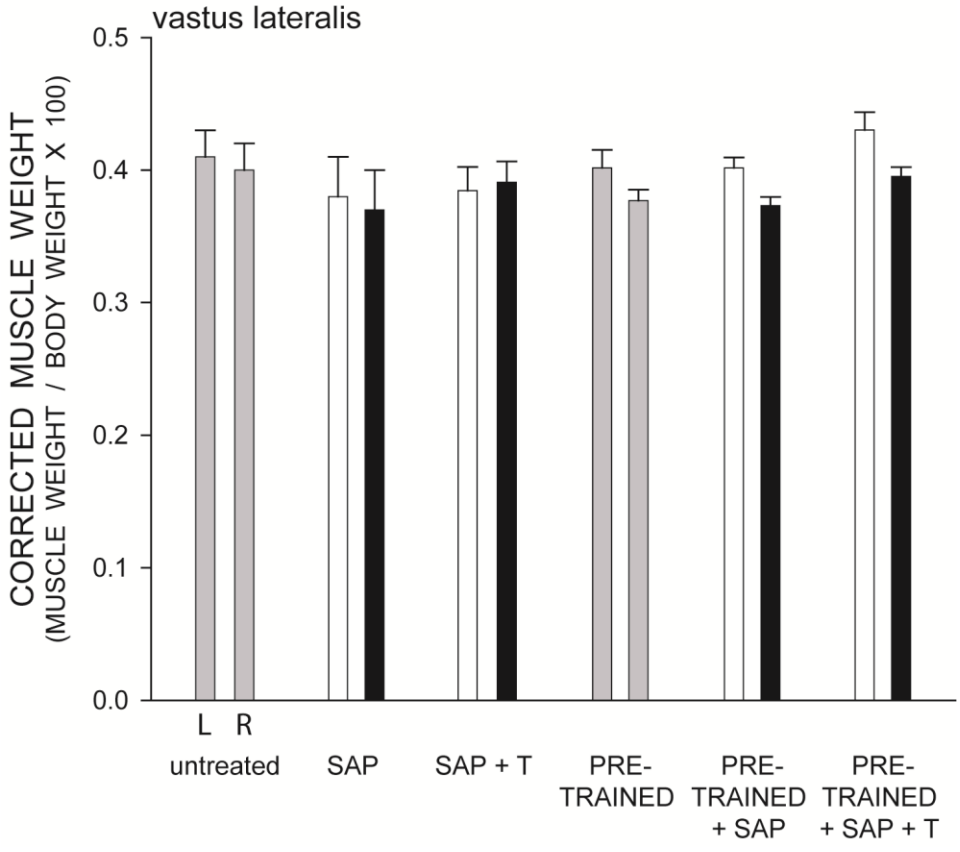

vastus lateralis
CORRECTED MUSCLE WEIGHT
(MUSCLE WEIGHT / BODY WEIGHT X 100)
0.5
0.4
0.3
0.2
0.1
0.0
L R
untreated
SAP
SAP + T
PRE-TRAINED
PRE-TRAINED + SAP
PRE-TRAINED + SAP + T

Figure 7.2. Weights of the vastus medialis and vastus lateralis muscles corrected by body weight in untreated animals and sedentary saporin-injected animals that either received no further treatment (SAP) or were given a Silastic testosterone implant (SAP+T), at four weeks after saporin injection. Also included are animals who were given *ad lib* exercise for four weeks and were either sedentary for four weeks (PRE-TRAINED), received saporin and no further treatment (PRE-TRAINED+SAP), or received saporin and a testosterone implant (PRE-TRAINED+SAP+T), at four weeks after saporin injection (if applicable). Gray bars represent weights from the right (R) and left (L) sides in untreated animals and pre-trained animals who did not receive saporin. Black bars represent weights from the untreated contralateral (right) leg, and white bars represent weights from the saporin injected (left) leg of saporin-injected animals. Saporin injection reduced the weight of the injected vastus medialis muscle; neither pre-training nor testosterone implantation alone had an effect on left vastus medialis weight in saporin injected animals. However, pre-trained saporin animals who also received a testosterone implant did show a minor hypertrophy of the left vastus medialis compared to saporin animals who received no further treatment. Saporin animals who exercised showed minor hypertrophy of the (right) vastus medialis contralateral to the saporin injected muscle. Weights of both the left and right vastus lateralis were not affected by saporin injection or exercise. Bar heights represent means ± SEM. * indicates significantly different from untreated animals. † indicates significantly different from untreated saporin-injected animals.

The effect of saporin injection on quadriceps weight was specific to the injected muscle. In untreated animals, the corrected weights of the right (0.40 ± .02) and left (0.41 ± .02) VL muscles were similar [paired t-test, $t(4) = .43$, *ns*]. The weights of the VL muscles on the untreated (right) side did not differ across groups [$F(5,27) = 0.78$, *ns*]. Most importantly, the weights of the VL muscles adjacent of the saporin-injected (left) VM muscles also did not differ across groups [$F(5,27) = 0.52$, *ns*].

Motoneuron Counts

In untreated animals, the number of motoneurons within the identified quadriceps range did not differ between the left (251.2 ± 14) and right (237.6 ± 25) motor column [paired t-test, $t(4) = 0.63$, *ns*]. Injection of saporin into the left VM resulted in the death of ipsilateral quadriceps motoneurons, significantly reducing the number of motoneurons in the left motor column relative to that of the right [Fig. 7.3; $F(5,23) = 5.58$, $p < 0.01$]. Unilateral injection of saporin into the left VM resulted in a 21% reduction in the relative number of motoneurons compared with that of untreated animals (LSD, $p < 0.02$). Neither pre-training nor hormone treatment prevented the saporin-induced reduction in motoneuron number (overall average of 27% reduced; LSDs, $p < 0.001$ compared to untreated animals).

Motoneuron Morphometry

Injection of BHRP successfully labeled quadriceps motoneurons in all groups (Fig. 7.4). The dendritic arbor of labeled quadriceps motoneurons was strictly unilateral, with extensive ramification along the ventrolateral margins of the gray matter and in the lateral funiculus, as well as throughout the ventral horn. An average of 25.03 (± 1.46) motoneurons per animal was labeled with BHRP, and this did not vary across groups [$F(5,23) = 1.40$, *ns*].

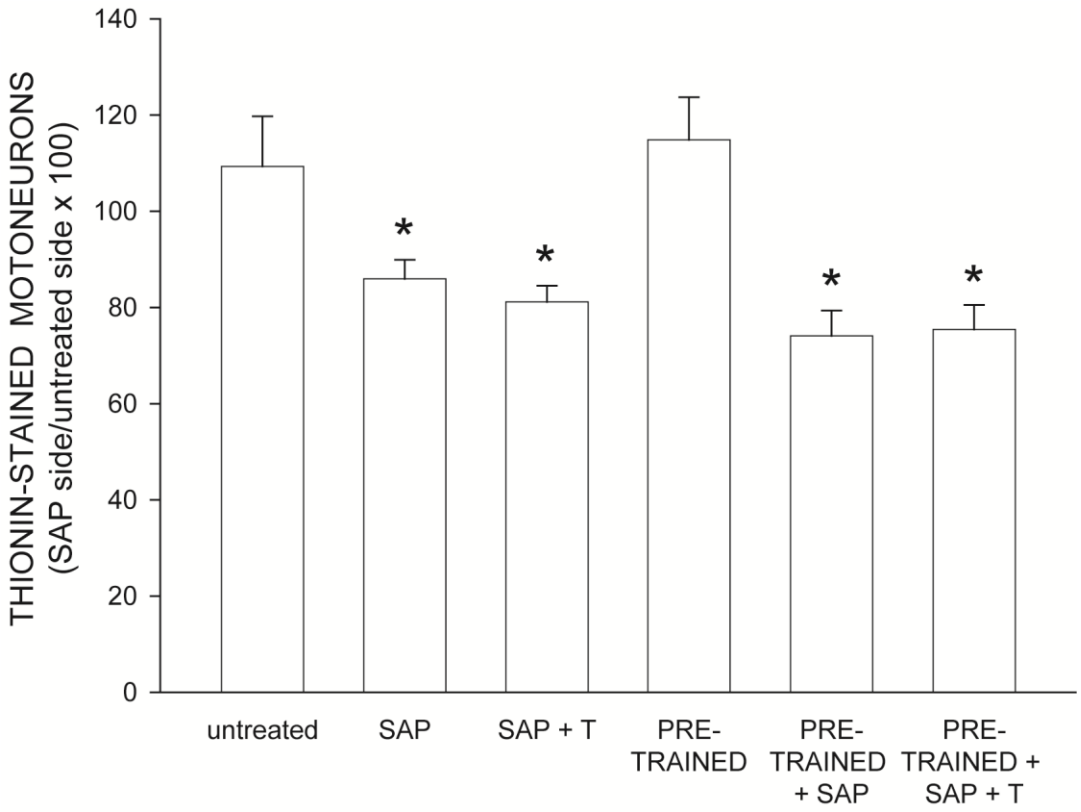


Figure 7.3. Numbers of quadriceps motoneurons in untreated animals and sedentary saporin-injected animals that either received no further treatment (SAP) or were given a Silastic testosterone implant (SAP+T), at four weeks after saporin injection. Also included are animals who were given *ad lib* exercise for four weeks and were either sedentary for four weeks (PRE-TRAINED), received saporin and no further treatment (PRE-TRAINED+SAP), or received saporin and a testosterone implant (PRE-TRAINED+SAP+T), at four weeks after saporin injection (if applicable). All bars are expressed as a ratio of motoneuron number ipsilateral to the saporin-injected muscle relative to that on the untreated side. Saporin killed approximately 27% of the ipsilateral quadriceps motoneurons, regardless of prior or subsequent treatment. Bar heights represent means ± SEM. * indicates significantly different from untreated animals.

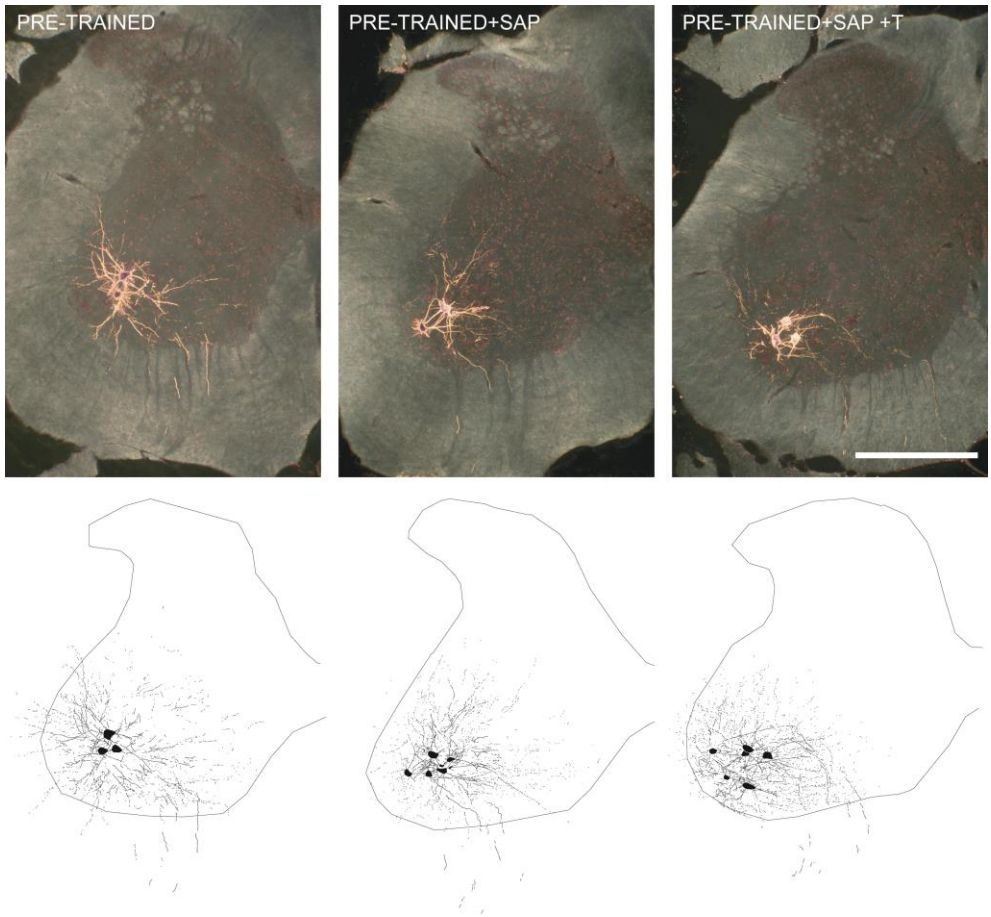


Figure 7.4. Darkfield digital micrographs of transverse hemisections through the lumbar spinal cords and computer-generated reconstructions BHRP-labeled somata and processes of animals who were given *ad lib* exercise for four weeks and were either sedentary for an additional four weeks (PRE-TRAINED), received saporin and no further treatment (PRE-TRAINED+SAP), or received saporin and a testosterone implant (PRE-TRAINED+SAP+T) after BHRP injection into the left vastus lateralis muscle. Micrographs and composites for untreated and saporin animals who either received no further treatment or a testosterone implant can be found in Chapter 1 (Fig. 1.1). Computer-generated composites of BHRP labeling were drawn at 480 μm intervals through the entire rostrocaudal extent of the quadriceps motor pool; these composites were selected because they are representative of their respective group average dendritic lengths. Scale bar = 500 μm.

Dendritic Length

Surviving quadriceps motoneurons underwent marked dendritic atrophy (Fig. 7.5). Dendritic length was decreased by 64% in saporin-injected animals who received no further treatment compared to that of untreated animals [LSD, $p < .0001$; overall test for the effect of group on dendritic length $F(5,23) = 5.4$, $p < 0.01$]. Compared to untreated animals, dendritic lengths were significantly shorter in all saporin-injected animals, regardless of pre-trained status or hormone treatment (overall average reduction of 54.3%; LSDs, $p < 0.001$).

Testosterone was effective in attenuating dendritic atrophy. Dendritic lengths of testosterone-treated saporin animals were 65% longer than those of saporin animals, but this difference failed to reach significance (LSD, $p = 0.07$). Similarly, dendritic lengths of pre-trained saporin animals who received testosterone were also longer (43% increase) than those of pre-trained saporin animals, but this difference also failed to reach significance (LSDs, $p = .23$). Pre-trained saporin animals who received testosterone were also longer than those of untrained saporin animals (71% increase), but this difference also failed to reach significance (LSDs, $p = .07$).

Pre-training was not able to attenuate dendritic atrophy in saporin-injected animals. Dendritic lengths in pre-trained saporin-injected animals were not significantly different compared to those of untrained saporin animals (LSD, $p = .61$). Pre-training also did not increase the efficacy of testosterone treatment in attenuating dendritic atrophy. Dendritic lengths of pre-trained saporin animals who received testosterone treatment were not significantly different than those of untrained saporin animals who received testosterone treatment (LSD, $p = .89$).

Interestingly, pre-trained animals who did not receive saporin showed shorter dendritic lengths compared to untreated animals (46% shorter than untreated; LSD, $p < 0.01$). Dendritic lengths of pre-

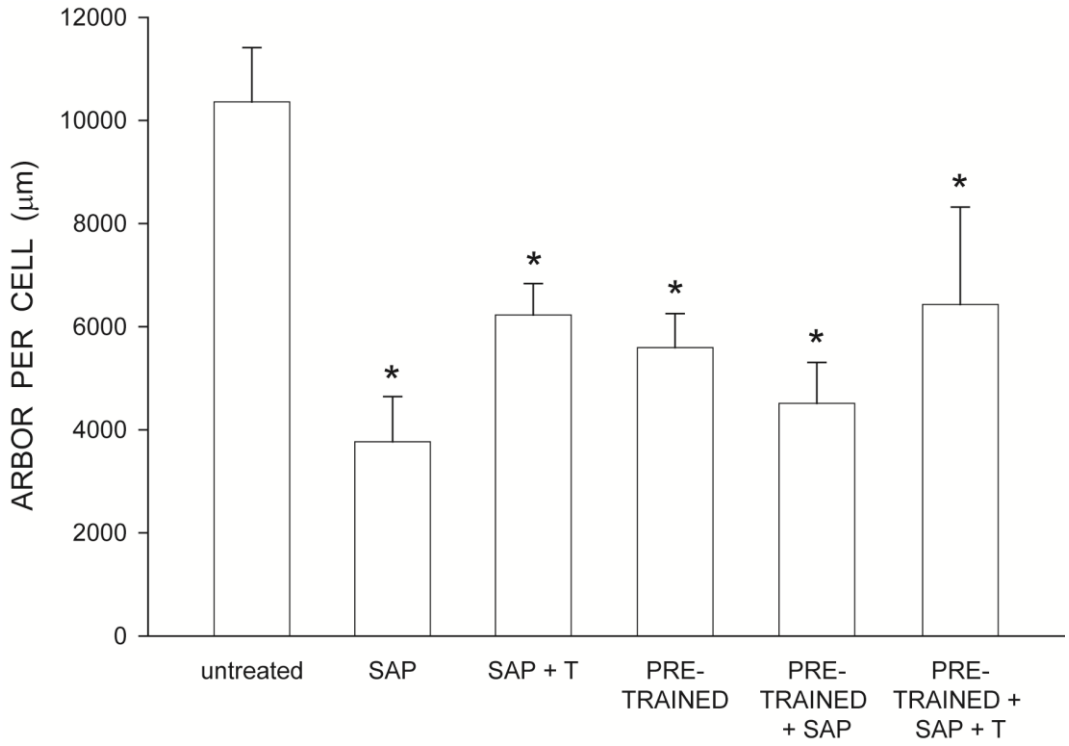


Figure 7.5. Dendritic lengths of quadriceps motoneurons in untreated animals and sedentary saporin-injected animals that either received no further treatment (SAP) or were given a Silastic testosterone implant (SAP+T), at four weeks after saporin injection. Also included are animals who were given *ad lib* exercise for four weeks and were either sedentary for an additional four weeks (PRE-TRAINED), received saporin and no further treatment (PRE-TRAINED+SAP), or received saporin and a testosterone implant (PRE-TRAINED+SAP+T), at four weeks after saporin injection (if applicable). Following saporin-induced motoneuron death, surviving neighboring motoneurons lost almost 64% of their dendritic length and testosterone treatment attenuated this dendritic atrophy. Pre-training was not effective in attenuating this dendritic atrophy, and did not alter the effectiveness of testosterone treatment in attenuating dendritic atrophy. Pre-trained animals who did not receive saporin also had dendritic lengths shorter than untreated animals. Bar heights represent means ± SEM. * indicates significantly different from untreated animals.

trained animals who did not receive saporin were comparable to animals who received saporin, regardless of training status (LSDs, *ns*).

Dendritic Distribution

Dendritic length was non-uniformly distributed across radial bins, and a repeated measures ANOVA revealed a significant effect of radial location [Fig. 7.6; $F(11,253) = 12.57$, $p < 0.0001$]. Consistent with the results seen in total dendritic length analysis, there was also a significant effect of group [$F(5,253) = 6.81$, $p < 0.001$]. There were reductions in dendritic length throughout the radial distribution, ranging from 38% (180º to 240º) to 79% (60º to 120º) in saporin-injected animals compared with untreated animals [$F(1,99) = 26.19$, $p < .0007$]. Testosterone treatment was effective in attenuating these reductions, with reductions ranging from 26% (300-360°) to 52% (120-180°) compared to untreated animals [$F(1,99) = 12.15$, $p < 0.01$]. Dendritic lengths per bin were longer in saporin animals who received testosterone treatment compared to saporin animals who received no further treatment [$F(1,110) = 6.63$, $p < .03$], with increases ranging from 8% (240-300%) to 136% (60-120°).

Pre-trained animals showed reductions in dendritic length in select portions of the dendritic distribution, with reductions in dendritic length ranging from 20% (180º to 240º) to 67% (60º to 120º) compared to untreated animals [$F(1,77) = 15.20$, $p < .001$]. Throughout the radial distribution, dendritic lengths per bin in were similar in pre-trained saporin-injected animals and those of untrained saporin-injected animals who received no further treatment [$F(1,88) = 0.09$, *ns*]; reductions ranged from 46% (240º to 300º) to 78% (120º to 180º) compared to untreated animals. Pre-trained animals who received saporin injection and testosterone treatment did not show any differences in dendritic distribution compared to untrained saporin animals who received testosterone treatment [$F(1,88) = 0.01$, *ns*]; reductions ranged from 11% (240º to 300º) to 60% (60º to 120º) compared to untreated animals.

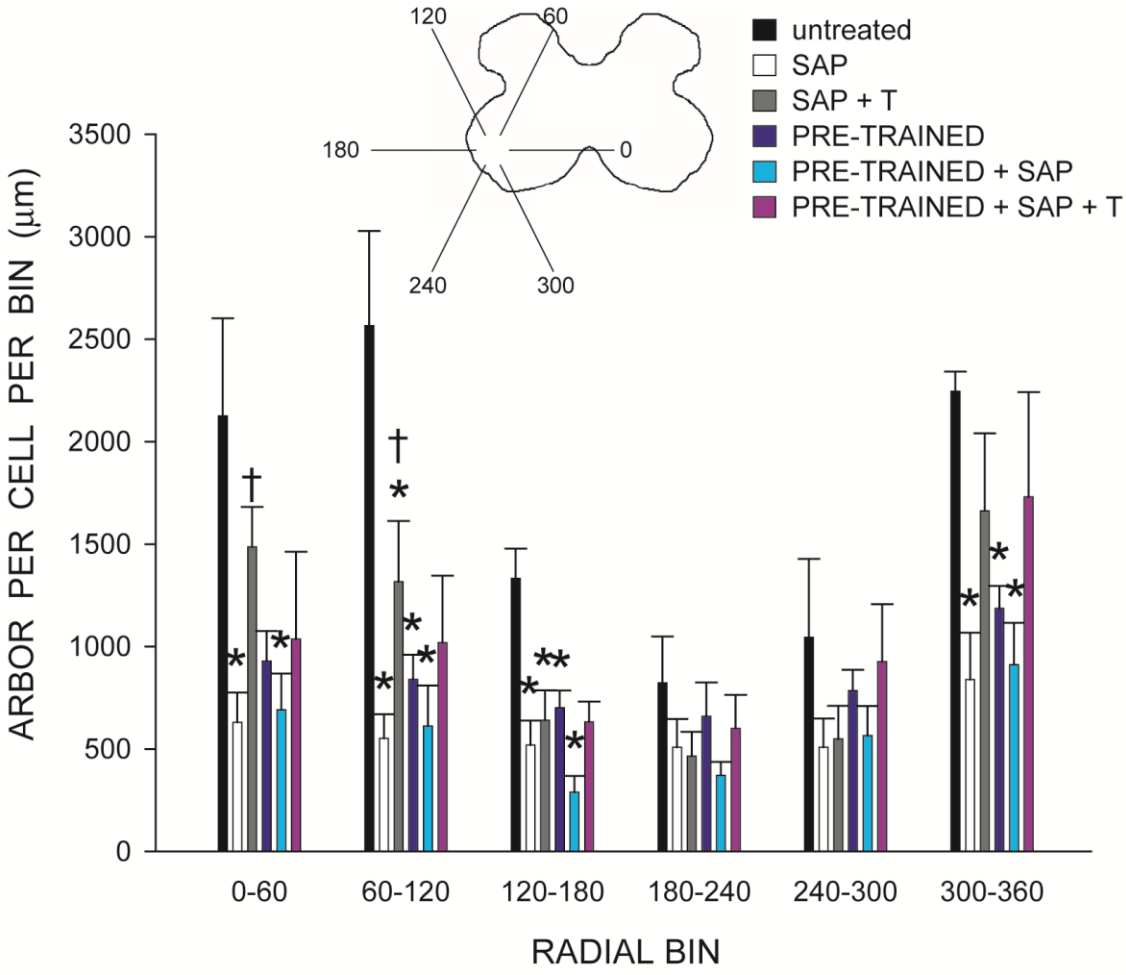

120
60
180
0
240
300
untreated
SAP
SAP + T
PRE-TRAINED
PRE-TRAINED + SAP
PRE-TRAINED + SAP + T
ARBOR PER CELL PER BIN (µm)
3500
3000
2500
2000
1500
1000
500
0
0-60
60-120
120-180
180-240
240-300
300-360
RADIAL BIN

Figure 7.6. Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron dendritic distribution. Length per radial bin of quadriceps dendrites in untreated animals (black bars) and sedentary saporin-injected animals that either received no further treatment (SAP; white bars) or were given a Silastic testosterone implant (SAP+T; gray bars), at four weeks after saporin injection. Also included are animals who were given *ad lib* exercise for four weeks and were sedentary for an additional four weeks (PRE-TRAINED; dark blue bars), received saporin and no further treatment (PRE-TRAINED+SAP; light blue bars), or received saporin and a testosterone implant (PRE-TRAINED+SAP+T; purple bars), at four weeks after saporin injection (if applicable). For graphic purposes, dendritic length measures have been collapsed into 6 bins of 60° each. Quadriceps motoneuron dendritic arbors display a non-uniform distribution, with the majority of the arbor located between 300° and 120°. Following saporin-induced motoneuron death, surviving neighboring motoneurons had reduced dendritic length throughout the radial distribution. Testosterone treatment, but not pre-training alone, attenuated this reduction. Pre-trained animals who did not receive saporin also showed reduced dendritic throughout the distribution. Bar heights represent means ± SEM. * indicates significantly different from untreated animals. † indicates significantly different from untreated saporin-injected animals.

Interestingly, dendritic length per bin in pre-trained animals who received no further treatment were not significantly different from either pre-trained animals who only received saporin [$F(1,66) = 2.34$, *ns*] or received saporin and testosterone treatment [$F(1,66) = 0.21$, *ns*]. Testosterone treatment did not affect radial dendritic distribution in pre-trained animals who received saporin [$F(1,66) = 1.57$, *ns*].

Dendritic Extent

In agreement with the nonuniform dendritic distribution of quadriceps motoneurons (Fig. 7.6), radial extent differed across bins (Fig. 7.7), and a repeated measures ANOVA revealed a significant effect of radial bin [$F(11,253) = 31.81$, $p < 0.0001$]. However, there was not a significant effect of group [$F(5, 253) = 2.17$, *ns*] on dendritic radial extent.

Rostrocaudal dendritic extent spanned 3776.0 ± 546.3 μm in untreated animals. Neither pre-training, saporin injection, nor testosterone treatment had an effect; rostrocaudal extent did not differ across groups [overall average 3531.0 ± 151.3 μm; $F(5,23) = 1.01$, *ns*].

DISCUSSION

Surviving motoneurons respond to the death of neighboring motoneurons with marked dendritic atrophy (Little et al., 2009). Treatment with either testosterone or exercise is protective against dendritic atrophy in surviving motoneurons (Litle et al., 2009; Chew and Sengelaub, 2019), and both treatments are mediated by classical receptor activation (Cai et al., 2017; Chapter 5). In Chapter 6 of this dissertation, we demonstrated that exercise upregulates androgen receptor expression in skeletal muscle. In this chapter, we hypothesized that the upregulation of androgen receptors at the target muscle after exercise would confer resilience to subsequent dendritic atrophy and increase the neuroprotective efficacy of testosterone treatment following partial motoneuron depletion. The data does not support this

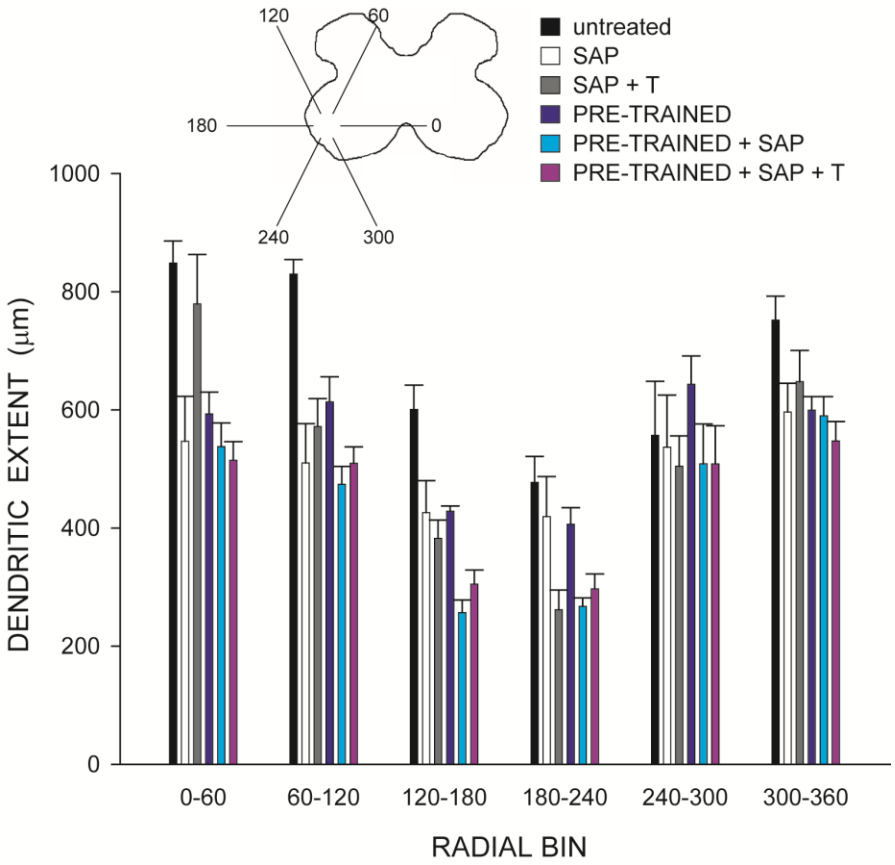


Figure 7.7. Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron radial dendritic extent. Radial extents of quadriceps dendrites in untreated animals (black bars) and sedentary saporin-injected animals that either received no further treatment (SAP; white bars) or were given a Silastic testosterone implant (SAP+T; gray bars), at four weeks after saporin injection. Also included are animals who were given *ad lib* exercise for four weeks and were either sedentary for an additional four weeks (PRE-TRAINED; dark blue bars), received saporin and no further treatment (PRE-TRAINED+SAP; light blue bars), or received saporin and a testosterone implant (PRE-TRAINED+SAP+T; purple bars), at four weeks after saporin injection (if applicable). For graphic purposes, dendritic extent measures have been collapsed into 6 bins of 60° each. There were no group differences in dendritic radial extent. Bar heights represent means ± SEM.

hypothesis; pre-training neither attenuated dendritic atrophy nor increased the efficacy of testosterone treatment. Interestingly, pre-trained animals who did not receive saporin showed reduced dendritic lengths compared to untreated animals, suggesting that cessation of exercise may cause dendritic atrophy.

Running wheel performance

All exercised animals in this experiment were allowed to exercise prior to any other manipulations. This led us to hypothesize that there would be no differences in cumulative distance run per group, and our hypothesis was supported.

Volume of exercise has been shown to have an effect on the hormonal response to exercise (Tremblay et al., 2005), with increased duration of exercise showing a threshold effect of a more pronounced increase in free testosterone during and shortly after exercise. Given that we have established that the mechanism of exercise-driven neuroprotection is androgen-dependent (see: Chapters 4 and 5), and our hypothesized mechanism of pre-training was due to upregulation of muscular androgen receptors, any substantial differences in the testosterone response following exercise was a potential confound. Thus, it was important to confirm that there was no difference in overall distance run across groups.

Saporin injection and muscle weights

Consistent with the results discussed in previous chapters, saporin injection into the VM reduced muscle weight and the number of innervating motoneurons. This induced death was specific to the motoneurons innervating the saporin-injected VM muscle; there were no changes in the number of BHRP-labeled motoneurons projecting to the ipsilateral VL. This remains an important consideration for interpreting the effects seen on the morphology of surviving motoneurons. The unchanged number of

motoneurons innervating the VL indicates that the changes in their dendritic morphology we observed (see below) cannot be due to accidental spread of saporin in the periphery (and subsequent death of VL motoneurons).

Neither pre-training nor testosterone treatment prevented saporin-induced decreases in the weight of the injected VM muscle, nor did they prevent saporin-induced motoneuron death. Despite the lack of protective effect observed, this confirmation of the saporin-induced death of motoneurons allows us to eliminate a potential confound that pre-training or hormone treatment attenuating either the degree of peripheral damage or the ability of saporin to kill motoneurons.

The present group difference in the weight of the uninjected (right) in VM animals allowed to exercise was previously observed in Chapters 4 and 5 but was not present in the results discussed in Chapter 3. One notable difference is that the experiments in Chapters 3-5 all required animals to exercise after saporin injection, while the current chapter required animals to run prior to receiving saporin. The presence of this hypertrophy of the right VM in this experiment, where exercise occurred prior to saporin injection, further corroborates the theory proposed in Chapters 4 and 5.Namely, that exercise and the accompanying increase in circulating testosterone are driving a compensatory hypertrophic effect in the right VM following the atrophy the left VM. The data reported in Chapter 6 showed that at four weeks after exercise, serum testosterone is increased compared to untrained animals, and that this elevation reverts towards baseline over the course of an additional four weeks of no exercise. Thus, this elevation in serum testosterone persists during the initial weeks of motoneuron death and accompanying muscular atrophy (Coons et al., 2009), and it is possible that the still-elevated concentration of serum testosterone is sufficient to drive the compensatory effect in right VM, despite not exercising after receiving saporin.

Interestingly, this hypertrophy of the right VM was present in all pre-trained groups and was exacerbated in the pre-trained saporin animals who received testosterone treatment. This suggests that the compensatory hypertrophy is testosterone dose-dependent; this phenomenon is discussed in greater detail in Chapter 8. There were no such group differences observed in the left VL. Thus, it remains unlikely that any changes in dendritic length are related to changes in innervated muscle weight.

Pre-training is not neuroprotective against motoneuron dendritic atrophy

Pre-training prior to partial motoneuron depletion was not effective in attenuating dendritic atrophy. Based on the results described in Chapter 6, we hypothesized that the increase in androgen receptor expression at the necessary site of androgen action (Chapter 5) would render the target muscle more sensitive to existing circulating androgens, thus attenuating dendritic atrophy following the death of neighboring motoneurons. The data do not reflect this; the dendritic lengths in pre-trained saporin animals were not significantly different from untrained saporin animals.

This lack of resilience in pre-trained animals may be explained by a number of possible mechanisms regarding the interactions between exercise, androgens and androgen receptors, and collateral dendritic atrophy. The foremost and most simple explanation is that pre-training is not sufficient to provide the neuroprotective effect seen with exercise after injury under any circumstances. This would suggest that exercise can be used to promote recovery from neural injury, but is ineffective as a prophylactic.

Another possible explanation is that exercise could confer resilience, but that the timing of the exercise protocol in relation to saporin injection in this experiment was insufficient to do so. Prior work in our lab has demonstrated that testosterone treatment is effective in attenuating dendritic atrophy (Little et al., 2009), and that this treatment can remain effective when constrained to the latter two weeks of the four week recovery period post-saporin injection (Coons and Sengelaub, 2008). The data

discussed in Chapter 6 indicates that while exercise does upregulate androgen receptor expression at the target muscle, the elevation reverts towards normal androgen receptor levels sometime in the four weeks after exercise is stopped. While we did not collect time series data that tracked the rate of androgen receptor expression across that four week sedentary period post-exercise, it is possible that the decrease in muscular androgen receptor expression over that time could push the number of androgen receptors beneath a necessary threshold above normal that is required to provide resilience.

However, a testing of this potential mechanism would prove extraordinarily difficult. Delaying the cessation of exercise until after the injection of saporin would introduce a damning confound, as testing whether exercise confers resilience innately requires stopping exercise prior to injury onset. Shortening the four week recovery period following saporin injection in order to provide less time for androgen receptor normalization after stopping exercise also raises serious confounds; all previously collected measures of dendritic length have used a four week recovery period and we have established that the effective window of testosterone-mediated protection is in the latter two weeks of a four week recovery period. This indicates that the androgen-mediated protection of dendrites occurs in the latter two weeks of recovery, and shortening recovery to maintain exercise-induced elevations of androgen receptors would thus shorten the androgenic protective window.

Although there were no significant differences in the dendritic lengths of pre-trained saporin animals and untrained saporin animals, there was an interesting difference between these two groups and their respective counterparts that did not receive saporin. Untrained saporin animals displayed dendritic lengths that were substantially reduced from untreated controls (64% decrease), and this difference was statistically significant ($p < .0001$). Conversely, dendritic lengths in pre-trained saporin animals were only marginally shorter than those of pre-trained animals that did not receive saporin (19% decrease; Fig. 7.4), and this difference was not statistically significant ($p = .50$). This shows that despite pre-

training not attenuating dendritic atrophy compared to untrained saporin animals, it was quite effective in attenuating dendritic atrophy when compared to similarly trained animals who did not receive saporin. This does raise the question of why pre-trained animals displayed shorter dendritic lengths compared to untreated controls (addressed below), but also implies that saporin-induced dendritic atrophy is not exacerbated by whatever caused the dendritic atrophy in pre-trained animals who did not receive saporin.

Pre-training does not increase neuroprotective efficacy of androgen treatment

Pre-training was not effective in altering the neuroprotective efficacy of exogenous testosterone treatment in attenuating collateral dendritic atrophy. This was particularly noteworthy, as we had previously demonstrated that the same dosage of testosterone treatment was able to completely prevent dendritic atrophy in the SNB (Fargo and Sengelaub, 2004a,b) compared to only attenuating dendritic atrophy in the quadriceps (Little et al., 2009), and we attributed this difference to the androgen-sensitivity of the SNB-BC/LA muscle complex. The lack of increased protective effect could be due to the previously discussed downregulation of muscular androgen receptors following the cessation of exercise, thus removing the necessary adaptation that was hypothesized to driven any increase in the protective effect.

As discussed above, four weeks of exercise upregulates androgen receptor expression in skeletal muscle (Chapter 6), which is the necessary site of androgen action for both exercise and exogenous testosterone treatment in protecting motoneuron dendrites from secondary atrophy (Chung, 2015; Chapter 5). In the four weeks following the cessation of exercise, the number of androgen receptors seems to revert towards normal levels of expression (Chapter 6), which would undermine this experiment's initial hypothesis: that the increased number of androgen receptors at the muscular site of androgen action would drive a greater protective effect of exogenous testosterone. Furthermore, the

effective window of testosterone treatment has been shown to be in the latter two weeks of the four week recovery period (Coons and Sengelaub, 2008). Thus, it is possible that during the necessary temporal window in which testosterone is acting to protect motoneuron dendrites (i.e., the two weeks prior to sacrifice, and two weeks after stopping exercise), androgen receptor expression and serum testosterone have decreased from the elevated levels found after four weeks of exercise, eliminating the exercise adaptation necessary for the theorized increased efficacy of testosterone treatment.

If true, this would imply that *sustained* exercise after injury could increase the efficacy of testosterone treatment. This could be easily tested with a group that either begins exercise solely after saporin injection (i.e., the exercise manipulation described in Chapters 3-5) and simultaneously receives testosterone treatment, or by pre-training rats prior to saporin injection (i.e., the pre-training paradigm described in this chapter) and allowing exercise to continue after saporin injection in addition to receiving testosterone treatment.

Pre-training may confer androgen sensitivity to somatic neuromuscular systems

Perhaps the most interesting finding in the present experiment is the dendritic atrophy in pre-trained animals who did not receive saporin. Prior to data collection, pre-trained animals were hypothesized to be no different from untreated animals, as we had previously shown that intact animals (i.e., did not receive saporin) who exercised did not show any differences in dendritic length compared to untreated animals (Chapter 3). However, the present data showed that the dendritic lengths in pre-trained animals were of comparable length to saporin animals who did not receive any further treatment. This would suggest that the cessation of exercise can cause dendritic atrophy in motoneurons of comparable magnitude as that seen in motoneurons whose neighbors had died.

This phenomenon closely resembles that observed by Huguenard and colleagues (2011). Normally, the motoneurons of the quadriceps are relatively insensitive to hormonal manipulation;

castration produces no significant changes in muscular, dendritic, or somal morphology (Huguenard et al., 2011). In contrast, the androgen-sensitive SNB-BC/LA complex shows muscle atrophy, dendritic retraction, and shrinking of the somata following castration, and these negative effects are reversed with testosterone treatment (Kurz et al., 1986). This difference in the androgen-sensitivity of these two neuromuscular populations has been hypothesized to be due to the difference in the number of androgen receptors at each respective target muscle (Huguenard et al., 2011). This hypothesis has been supported by data showing that transgenic upregulation of the number of androgen receptors in the VL confers androgen sensitivity to previously insensitive quadriceps motoneurons. In rats genetically engineered with a human skeletal actin promoter that drove expression of androgen receptors in skeletal muscle (HSA-AR Tg), somatic skeletal musculature expressed androgen receptors at 3.8x the normal amount (Niel et al., 2009). These HSA-AR Tg rats show androgen-sensitive phenotypes in their VL motoneurons. Following castration HSA-AR Tg VL motoneurons undergo dendritic retraction and testosterone replacement prevents the retraction (Huguenard et al., 2011). This demonstrates that whether a motoneuron population is sensitive to hormonal manipulation is a plastic quality driven by the density of androgen receptors in the target musculature.

This principle can also be applied to the present study, and I propose that exercise is able to confer androgen-sensitivity to a previously insensitive motoneuron population. Exercise upregulates androgen receptor expression in the vastus lateralis, in similar fashion as the HSA-AR Tg. A key difference between these two sources of induced androgen sensitivity lies in the transient nature of the exercise-induced upregulation compared to the permanent HSA-AR Tg alteration [Huguenard and colleagues (2011) did not use an inducible means of genetic manipulation; e.g., Cre-Lox recombination]. Because the HSA-AR Tg upregulation of VL androgen receptor persists throughout the animal's lifetime, castration or other manipulations (e.g., flutamide) are necessary to deprive the neuromuscular complex of androgen signaling and induce dendritic retraction (Huguenard et al., 2011). In contrast,

androgen receptor elevation reverts towards normal levels of expression within four weeks following the cessation of exercise. Thus, in the current study's pre-trained group, stopping exercise may be sufficient to decrease androgen signaling, driving decreased expression of androgen receptors, which could act as an analog to castration, causing dendritic retraction.

This androgen receptor dependent regulation of motoneuron dendritic structure is likely rooted in an interaction of androgens and neurotrophins, particularly brain-derived neurotrophic factor (BDNF), which are known to work together to regulate motoneuron structure (Verhovshek and Sengelaub, 2010). Castration normally causes atrophy of SNB motoneuron dendrites (Verhovshek and Sengelaub, 2010) and alters the expression of BDNF in both the BC/LA muscles and the SNB motoneurons (Verhovshek et al., 2010). Conversely, castration does not cause atrophy of quadriceps motoneurons (Huguenard et al., 2011), but does cause a decrease in BDNF immunostaining in both the VL and innervating motoneurons (Verhovshek et al., 2010). The key difference arises in how BDNF expression is altered at each observed site following castration. BDNF expression is decreased in both motoneuron populations (the SNB and quadriceps motoneurons), but is differentially affected at the peripheral musculature; BDNF decreased in the VL but increased in the BC/LA. Clearly, the androgen sensitivity of the SNB-BC/LA confers some quality that causes this difference in BDNF expression at the target musculature following castration, and this is hypothesized to be the molecular manifestation for why the two motoneuron populations show different responses following castration.

This difference in the target neurotrophin expression between these two neuromuscular systems is yet another link in the mechanism that explains their different neuroplastic qualities as a result of their hormonal sensitivity. The results of the present experiment provide the framework for an interesting future experiment that could determine whether the proposed conferral of androgen sensitivity via

exercise also causes a corresponding change in the BDNF expression, either at the target muscle or the motoneuron population, following the cessation of exercise.

CONCLUSIONS

Chapters 3, 5, and 6 of this thesis established that exercise is neuroprotective on motoneuron dendrites following the induced death of their neighbors (Chew and Sengelaub, 2019), that the protective effect is dependent on androgen action at the target muscle (Chapter 5), and that the same exercise protocol upregulates androgen receptor expression at the target muscle (Chapter 6). Here we tested whether pre-training with the same exercise protocol prior to neural injury could provide resilience to dendritic atrophy or increase the effectiveness of exogenous testosterone treatment. Our findings indicate that pre-training neither confers resilience nor modulates the effectiveness of supplemental testosterone treatment in attenuating dendritic atrophy in motoneurons, but that pre-training alone can induce dendritic atrophy. I believe that this atrophy following pre-training is reflective of induced androgen sensitivity by exercise, and is related to the decrease in muscular androgen receptor expression in pre-trained animals compared to exercised animals. If true, this would imply that behavioral manipulations that affect gonadal hormones, such as exercise, can produce plastic changes in the hormonal sensitivity of somatic motoneuron populations.

CHAPTER 8

UNIFIED DISUCSSION

The purpose of this dissertation was to investigate the following questions: 1) whether exercise can be neuroprotective to spinal motoneurons following adjacent motoneuron death, 2) whether exercise is neuroprotective via the same mechanism as previously established hormonal therapeutics, and 3) whether prior exercise causes physiological adaptations that can alter the efficacy of hormonal therapeutics. These questions are individually examined in greater detail in Chapters 3-7 of this dissertation. In this final chapter, I will summarize the findings of the previous chapters, will elaborate on the larger implications of the investigated mechanism, and will provide suggestions for future avenues of related research.

Summary of prior and current findings

Previous work in our lab has demonstrated that spinal motoneurons are negatively affected by the death of their neighbors (Fargo and Sengelaub, 2004a,b; Little et al., 2009). The dendritic arbors of surviving motoneurons undergo retraction, losing approximately 60% of their original length. This atrophy results in a loss of synaptic connections and accompanying decrease in functionality (Little et al., 2009). Both the atrophy of dendritic arbor and decreases in functional excitability can be attenuated, or even prevented altogether in some neuronal populations, by treatment with testosterone (Fargo and Sengelaub, 2004a,b; Little et al., 2009). Testosterone acts specifically at the target muscle of the observed motoneurons by binding to the androgen receptor, demonstrating a mechanism requiring classical steroid receptor activation at a necessary site of action (Chung, 2015; Cai et al., 2017).

The present studies have established the following novel findings. Chapter 3 provided evidence to support exercise is neuroprotective to the dendrites of surviving quadriceps motoneurons, and to a degree comparable to that seen with exogenous hormone treatment. Chapters 4 and 5 demonstrated that these neuroprotective effects of exercise are also dependent on androgen action, utilizing the same mechanism that specifically requires androgen receptor activation at the target muscle. This indicates that both exercise and hormone treatments utilize similar biological mechanisms in order to influence motoneuron morphology, in this case in a protective fashion. Subsequent analysis also revealed no correlation between the volume of exercise and the degree to which surviving motoneuron dendrites were protected from atrophy following the death of neighboring motoneurons.

Exercise also induces a number of physiological adaptations, and Chapter 6 established that these adaptations include alterations to expression of the androgen receptor at the site of neuroprotective androgen action, the target muscle of the observed motoneurons. Chapter 7 investigated whether these adaptations were sufficient to either induce resilience to subsequent injury or to increase the efficacy of hormonal treatment in attenuating dendritic atrophy, but the data suggests that neither of these hypotheses were correct. Chapter 7 also demonstrated that exercise confers androgen sensitivity to typically insensitive somatic neuromuscular systems.

Despite this dissertation's primary focus on assessment of dendritic structure, our other collected measures also confirm several key findings. Cell count data of quadriceps motoneurons in the left and right ventral horns indicated that unilateral injection of saporin conjugated to the cholera toxin B-subunit (CTB-saporin) into the vastus medialis (VM) was successful in killing motoneurons in only in the ipsilateral quadriceps motor pool. While this is unsurprising and is consistent with other studies using CTB-saporin to induce motoneuron death (Fargo and Sengelaub, 2004a,b; Little et al., 2009; Chew et al., 2019), this does provide evidence that exercise is not able to attenuate saporin-induced motoneuron

death via ribosome inactivation (Stirpe et al., 1983, 1987). While we cannot extrapolate with certainty based on the present data, I believe that this is evidence that exercise is not sufficient to prevent many forms of pathological cell death. In applying this principle to disease, this would be akin to saying that exercise is not able to prevent the primary pathology of amyotrophic lateral sclerosis (ALS), but rather that exercise is able to attenuate some of the secondary pathologies (i.e., dendritic atrophy in surviving motoneurons) that emerge from the primary pathology (i.e., the death of some motoneurons). Thus, while exercise is beneficial in the larger goal of treating problems that emerge during neurodegenerative disease, it remains imperative to continue research into the ultimate cause of why neurons die in specific disease, so that the initial problem of neuron death can be addressed.

The effects of exercise on skeletal muscle weight

The current series of exercise-focused experiments saw the emergence of a previously unseen group difference in the weights of the right VM, the contralateral analog to the saporin-injected muscle in all saporin groups. The right VM normally is unaffected by saporin injection, and the measure of its weight is collected as confirmation that there are within-subjects differences in VM weight between saporin-injected and non-injected muscles. This lack of effect on right VM weight was present in all of our prior studies on investigating saporin injection into the quadriceps (Little et al., 2009; Cai et al., 2017; Chew et al., 2019), including the initial experiment examining whether exercise is neuroprotective to quadriceps motoneuron dendrites (Chew and Sengelaub, 2019). However, there was a significant group effect on right VM weight in all of our subsequent studies examining exercise effects on quadriceps motoneurons in which muscle weight data was collected (Chapters 4, 5, and 7). This was puzzling. In Chapter 4, I speculate that this hypertrophy could be an exercise-dependent response to the saporin-induced reduction of the left VM, with the right VM compensating for the contralateral

reduction in force production and weight bearing of the left VM (Tsumiyama et al., 2014), but this speculative explanation does not explain why the effect was not statistically significant in Chapter 3.

Our current hypothesis is that there was an interaction of exercise and saporin injection, and the addition of more groups that received both saporin and exercise, regardless of other additional manipulations, was able to push the analysis over the threshold of statistical significance in Chapters 4, 5, and 7. The experiment in Chapter 3 examined two exercise groups, one that received saporin and another of intact animals. Thus, in Chapter 3, there were not a sufficient number of animals who received both saporin and exercise to produce an overall effect of group. However, Chapters 4, 5, and 7 all contain at least two groups that received both saporin and exercise, in addition to other manipulations. The persistence of this hypertrophy in the right VM in exercised saporin groups, and the lack of difference between exercised groups regardless of castration or androgen receptor blockade, lends credence to my hypothesis.

The mechanism that drives this compensatory hypertrophy of right VM is not entirely clear. My initial hypothesis was that the resulting increase in serum testosterone following exercise was driving the hypertrophy. Data from Chapter 6 demonstrated that both exercised and pre-trained animals had increased serum testosterone compared to sedentary animals, and androgens drive hypertrophy in male skeletal muscles in a dose-dependent manner (Bhasin et al., 1996; Bhasin et al. 2001b). All exercised rats showed the right VM hypertrophy compared to sedentary rats, and pre-trained rats who received saporin and a testosterone implant – the only exercised group that also received testosterone implantation – showed a greater increase in right VM mass compared to all other exercised rats. This seems to provide strong evidence that exercise is driving compensatory hypertrophy of the right VM through a dose-dependent androgenic mechanism.

However, this hypothesis is complicated by the persistence of the right VM hypertrophy in the exercised castrates (Chapter 4). The purpose of the orchiectomy procedure is to deprive the animal of the necessary organs to produce the vast majority of its endogenous testosterone. The effectiveness of this ablation of endogenous testosterone production was confirmed by assessment of the androgen-sensitive bulbocavernosus/levator ani muscle complex, which showed the expected atrophy after castration. Thus, it seems that the hypertrophy of the right VM in exercised animals after castration may be due to a mechanism that is not dependent on systemic androgens.

It is possible that the effect could still be androgenic in nature, but that it does not rely on androgens produced in the testes. There are studies that have reported the presence and activity of enzymes responsible for androgen synthesis located within skeletal muscle (Aizawa et al., 2007, 2008) and that the examined steroidogenic machinery is upregulated following exercise (Aizawa et al., 2010). A simple experiment to determine whether local androgen action is responsible for the hypertrophy of the right VM following saporin injection into the left VM would be to suture a local hydroxyflutamide implant, identical to those used in Chapter 5, to the right VM at the time of saporin injection. If the muscle does not hypertrophy in animals who receive such implants, then it can be assumed that the mechanism is androgenic. If the muscle does hypertrophy despite the implant, then it can be assumed that the mechanism is not androgenic.

Androgenic modulation of motoneuron structure

Prior results from our lab have established an androgenic neuroprotective mechanism in our CTB-saporin model of partial motoneuron depletion, specifically requiring cognate steroid receptor activation at the target muscle. This has been reiterated in this dissertation *ad nauseum*, but remains important. These previous findings established a neuroprotective mechanism that is the foundation for the experiments in this dissertation, and were among many links in an intellectual progression in the

overarching study of how hormone action can modulate motoneuron structure (e.g., Little et al., 2009; Cai et al., 2017). Those findings, in turn, were heavily influenced by work examining hormonal regulation of neuroplastic phenomena in both development and adulthood (e.g., Kurz et al., 1986; Goldstein et al., 1993). These initial findings of basic research from decades past have borne intellectual fruit that were critical in the motivating this dissertation.

The most robust findings of this dissertation are the establishment of a mechanism of how exercise attenuates secondary atrophy in motoneurons following the death of their neighbors and demonstrating that this mechanism is seemingly the same of that seen in supplemental hormone treatment. Both exercise and hormone treatments are dependent on classical steroid receptor activation, and this receptor activation is necessary specifically at the target muscle of the innervating motoneuron population of interest. The results from Chapters 3-5 demonstrate these phenomena in a sequentially more specific manner. Chapter 3 shows that exercise is neuroprotective, Chapter 4 shows that said protection is dependent on the presence of testes (likely through androgen secretion), and Chapter 5 shows that androgen receptor activation specifically in the target muscle of the motoneurons is necessary for the protective effect. Taken together, these results paint a more complete picture than any in isolation. For example, the isolated results from Chapter 4 do not address how orchidectomy contributes to the lack of protection of motoneuron dendrites in exercised rats. It is possible that non-androgenic effects stemming from the surgical procedure or the aforementioned decrease in early running behavior in castrated rats could have rippling effects that prevent exercise-driven neuroprotection. However, when these results are interpreted in tandem with those of Chapter 5, where rats had normal androgen production and running behavior was unaffected, the androgenic mechanism of neuroprotection by exercise is strengthened.

While the nature of this particular application of neuroprotection may seem hyper-specific and somewhat limited in its use, there are underlying qualities of the mechanism that are of particular interest. By discussing how different experiments contribute to the larger picture, we can parse out these qualities, speculate as to how they can be harnessed, and raise new questions to be addressed in future research.

The experimental chapters of this dissertation can be divided into two major categories: Chapters 3-5 establish that exercise is neuroprotective via the same mechanism used by supplemental testosterone treatment, while Chapters 6 and 7 examine whether the physiological adaptions produced by exercise can produce plastic changes to the neuroprotective mechanism that drives neuroplastic changes. Taken together, we can infer the following broad conclusions from the results: 1) a behavioral intervention can have the same effects as exogenous hormone treatment, via the same biological substrates, 2) in addition to the direct neuroprotective benefits, said behavioral treatment can induce plastic changes to the neuron-target tandem, and 3) these plastic changes can confer new qualities to that neuromuscular system.

The first of these points is interesting, but not particularly surprising. Exercise has a well-documented relationship with androgen action (Kraemer et al., 1995; Bhasin et al., 1996; Tremblay et al., 2004; Kraemer and Ratamess, 2005), and some neurotherapeutic effects of exercise have previously been demonstrated to be dependent on androgens (Thompson et al., 2014). The latter two points are far more interesting, as they highlight the malleability of physiology and the reciprocal influences that neurons and their targets have over each other, and open possible avenues of investigation into how far plastic changes to either component of the neuromuscular complex can ripple.

For example, results from Chapter 7 show that pre-trained rats who do not receive saporin show atrophy of quadriceps motoneuron dendritic arbors that is akin to the atrophy seen following castration

in an androgen-sensitive transgenic quadriceps (Huguenard et al., 2011). This data, interpreted within the context of the upregulation of androgen receptor expression found in Chapter 6, provides evidence that exercise can confer androgen sensitivity in a typically non-sensitive system. A more broad interpretation of these findings is that a simple behavior such as exercise was able to confer a transient qualitative change to the neuromuscular system. This raises many interesting questions about the adaptive effects of exercise (see below).

Future directions of research

Sex differences in exercise behavior and neuroprotection

One notable shortcoming in the studies comprising this dissertation, and an important consideration in extrapolating the findings, was the use of only male animals. Our lab has established that female rats also display reductions in total dendritic arbor length in surviving quadriceps motoneurons following the death of their neighbors, and that supplemental testosterone is effective in attenuating dendritic atrophy (Wilson et al., 2009). Interestingly, the same dose of testosterone that is effective in attenuating secondary dendritic atrophy in males is more effective in females. After partial motoneuron depletion with saporin, dendritic lengths of surviving testosterone-treated females are not significantly different from those of normal females (Wilson et al., 2009), while in males they are shorter than normal lengths but longer than saporin animals who receive no therapeutic treatment (Little et al., 2009). It is not entirely clear why there is a sex difference in the neuroprotective efficacy of the same testosterone treatment, but this difference likely reflects the females' response to the relatively larger than normal circulating level of testosterone. More relevantly, we have not determined whether exercise is neuroprotective following partial motoneuron depletion in females.

Exercise has been shown to be neurotherapeutic in both sexes following axotomy. Two weeks after axotomy and graft repair of the fibular nerve, regenerating axon profiles of mice given treadmill

training are longer in both males and females compared to their sedentary counterparts (Wood et al., 2012). Wood and colleagues also identified an important sex difference in the exercise regimens and facilitation of axon regeneration. Regenerating axons of males were only longer than those of sedentary controls if the males were allowed to walk on the treadmill in a slow, continuous manner for one hour (Wood et al., 2012). In contrast, regenerating axons in females were only longer than sedentary controls if the mice were subjected to an interval training paradigm requiring short, intense bursts of near-maximum running speed for 35 minutes of total training time (i.e., bursts of sprinting; Wood et al., 2012). Importantly, neither sex showed enhancement of axon regeneration when exposed to the alternate exercise protocol; males were unaffected by interval training and females were unaffected by slow, continuous running (Wood et al., 2012). The enhancement of axon regeneration following exercise was also shown to be androgen dependent, with the male effect disappearing with castration prior to exercise training (Wood et al., 2012) and the effect disappearing in both sexes with simultaneous flutamide treatment (Thompson et al., 2014).

Future studies could examine whether the previously observed differences in effective neurotherapeutic exercise protocols translate to neural injury models other than axotomy, including partial motoneuron depletion. If the interval training protocol is effective in attenuating secondary dendritic atrophy due to partial motoneuron depletion in females, future studies could examine whether the same androgenic mechanism observed in males is also responsible for neuroprotection in females. This would be a particularly interesting line of research, as exogenous testosterone treatment has been shown to be effective in females (Wilson et al., 2009), but females obviously do not produce testicular androgens that we have hypothesized to be responsible for the exercise-induced changes in androgenic signaling. Such a line of work could either establish that non-gonadal sources of androgens (i.e., the adrenal gland) could be subject to exercise effects, or establish a mechanism of exercise-driven neuroprotection that is not dependent on gonadal hormones. However, data collected to-date seems to

suggest that the androgenic mechanisms of exercise-driven neuroprotection treatment that I have described in males is also present in females, but with a few key differences.

Continuous exercise causes a major upregulation in male circulating androgen concentrations (Chapter 6; Wood et al., 2012), and castration prevents this upregulation (Wood et al., 2012). However, in females, neither continuous exercise nor interval training causes an upregulation of serum testosterone concentrations (Wood et al., 2012). This raises the question of how exercise enhancement of axon regeneration can be androgen-dependent (Thompson et al., 2014) without an accompanying increase in circulating androgen concentrations. The answer appears to not lie in systemic increases of androgens, but rather in local concentrations of hormone and hormone-metabolizing enzymes. Analysis of testosterone concentrations locally in skeletal muscle show that both males and females see increases in testosterone following exercise (Aizawa et al., 2008), and that females, but not males, show a decrease in the expression of P450 aromatase, an enzyme that converts testosterone into estradiol (Aizawa et al., 2008). This indicates that exercise results in the increase in muscular testosterone following exercise by a distinct mechanism in each sex. Males see an upregulation in the testicular production of androgens. In contrast, females cannot upregulate testicular androgen production, but compensate by decreasing aromatase activity to reduce the amount of testosterone metabolized, increasing the amount available for androgenic activity.

This is strong evidence that there is a sex difference in the mechanism of upregulating androgen signaling as a response to exercise. This is a fascinating example of both a conserved mechanism of hormonal neuroplasticity, and also the necessary differences in the mechanism necessitated by different sexual and endocrine anatomy. Further examination of such differences may also further broaden our understanding of therapeutics, such as why males and females require different training paradigms to see neuroprotective phenotypes.

Additional similarities with exogenous hormone treatment

A major finding of this dissertation was that exercise and exogenous hormone treatment are neuroprotective to spinal motoneurons via a common mechanism of androgen action at the target musculature. This seems to indicate that exercise is utilizing hormone to induce neuroprotection in the same manner as supplemental hormone treatment; however, this is not definitive proof that exercise and hormone supplementation are completely interchangeable as treatments. For instance, we have determined that testosterone treatment must be administered within a defined temporal window in order to be neuroprotective to motoneuron dendrites (Coons and Sengelaub, 2008). Normally, rats injected with saporin are allowed a four week recovery period before sacrifice. During this four week period, dendritic lengths atrophy, reach a nadir, and begin to recover towards normal lengths (Coons et al., 2009). Treatment with testosterone both prevents dendrites from reaching this normally observed nadir and accelerates their recovery towards normal lengths in a dose-dependent manner (Coons et al., 2009). Thus, testosterone is acting both as a protective agent (i.e., it protects dendrites from atrophying to their nadir) and a pro-regenerative agent (i.e., it promotes recovery and proliferation of dendritic length towards normal). Furthermore, testosterone is effective only when administered within a specific temporal window during that four week recovery period. When administered only during the first two weeks of the four week recovery, dendritic lengths at four weeks after saporin are not different from those of animals who did not receive any treatment (Coons and Sengelaub, 2008). However, rats who receive testosterone only during the latter two weeks of the four week recovery show dendritic lengths that are the same as rats treated during the entire four weeks (Coons and Sengelaub, 2008). This indicates that testosterone is effective in accelerating dendritic recovery only during the latter two weeks of recovery.

It remains to be seen whether exercise is subject to a similar temporal window, and also whether exercise affects dendritic structure in the same manner as testosterone across the entire four week recovery period. The studies in this dissertation only examined dendritic lengths at four weeks after saporin injection, which does not provide insight as to whether the dendrites follow the same atrophy and proliferation seen following saporin-injection with no further treatment or during testosterone treatment. It is possible that exercise is effective in attenuating both the initial atrophy and promoting recovery towards normal lengths in the exact same manner as testosterone treatment, but this remains unanswered. Furthermore, testosterone is neuroprotective in a dose-dependent manner (Coons et al., 2007); as dosage of testosterone increases, recovery of dendritic length towards normal also accelerates. While rats were allowed to run *ad lib* in Chapters 3-5 and 7, it is possible that there could be a necessary threshold of exercise in order to protect motoneuron dendrite from atrophy. Conversely, it is also possible that either overtraining or involuntary exercise are powerful enough stressors to either prevent or exacerbate dendritic atrophy. Thus, future studies could examine whether neuroprotection via exercise has either a restricted temporal window of treatment or necessary exercise volume requirements.

In addition to any potential similarities in timing or dose-dependency, exercise and exogenous testosterone treatment may share downstream molecular substrates that contribute to the protection of dendritic morphology. In Chapters 5 and 7, I discuss that androgen receptors at the target muscle and brain-derived neurotrophic factor (BDNF) interact to maintain innervating motoneuron dendritic morphology (Verhovshek and Sengelaub, 2010), with phenotypes of BDNF expression and changes to dendritic length that differ in androgen-sensitive and more conventional neuromuscular systems (Verhovshek et al., 2010). However, we currently do not have direct evidence that this interaction between androgens and BDNF is responsible for the neuroprotective effect of either exercise or

exogenous hormone treatment on motoneuron dendrites following saporin-induced partial motoneuron depletion.

Data from Chapter 6 and several other studies (Kraemer et al., 1991; Wood et al., 2012) demonstrate that exercise upregulates circulating and local muscular concentrations of testosterone (Aizawa et al., 2008). If exercise and hormone supplementation are, in fact, using the same mechanism of action in which increased androgen receptor activation at the target muscle causes corresponding changes in BDNF expression that protect dendritic length, then inhibition of BDNF signaling throughout the exercise or hormone treatment period should fail to attenuate dendritic atrophy in both interventions.

Simultaneous treatment with exercise and hormones

The experiment covered in Chapter 7 was designed in order to determine whether prior exercise would be able to confer either protective resilience to subsequent injury or increase the magnitude by which testosterone attenuates dendritic atrophy. Both of these hypotheses were motivated by the exercise-induced upregulation of the androgen receptor (Chapter 6), and both hypotheses were not supported by the data collected. I have noted that this could be due one or more of many possible factors. In Chapter 7, I specifically discuss whether the decrease in muscular androgen receptor expression in animals who were sedentary for four weeks after the cessation of exercise (i.e., pre-trained animals) compared to animals who were actively exercising could be the reason as to why pre-training is not effective in either conferring resilience or increasing the effectiveness of testosterone treatment. In brief, the number of muscular androgen receptors decreases in the pre-trained animals once they are sedentary, and the hypothesized necessary increased sensitivity to androgens is no longer present, thus explaining why testosterone treatment is not more effective in pre-trained animals.

While prior exercise was not able to increase the efficacy of subsequent testosterone treatment, it is not known whether simultaneous treatment with exercise and testosterone is able to produce

synergistic effects that protect motoneuron dendrites to a greater degree than either treatment alone. Given that we have demonstrated that the muscle is the necessary site of androgenic neuroprotection and that androgen receptors are upregulated during exercise, it is possible that simultaneous exercise and testosterone may protect motoneuron dendrites to a greater degree than either treatment alone.

Exercise adaptations

Chapter 7 was unsuccessful in demonstrating that prior exercise is able to confer resilience to subsequent injury. While the initial hypothesis was not supported, there are additional variations on the possible timing and history of exercise and whether they modulate neuroprotection to motoneuron structure. For example, the study design of Chapter 7 required rats to stop exercising after four weeks, at which time rats were injected with saporin. The reasoning for this was two-fold: 1) all prior studies in this line of work had used a four week exercise protocol, and 2) we were specifically investigating whether a history of exercise was able to confer resilience to injury or alter the neuroprotective efficacy of testosterone. Further studies could alter the duration of the exercise protocol to continue after the injection of saporin, and determine whether a history of exercise, or more fit physical state, alters the neuroprotective efficacy of *continued* exercise. The results of Chapter 5 demonstrated that androgen action at the target muscle is necessary for neuroprotection by exercise, and Chapter 6 demonstrated that androgen receptor expression in skeletal muscle is elevated to a significantly greater degree immediately after exercise when compared to pre-trained animals that were sedentary for four weeks after their previous four weeks of exercise. Thus, it is possible that continuing to exercise after an established history of exercise could maintain the elevation in androgen receptor at the target muscle, which could increase the neuroprotective efficacy of exercise.

Furthermore, we also observed that the dendritic lengths of quadriceps motoneurons in pre-trained animals that did not receive saporin were considerably shorter than those of untreated, sedentary

animals. We hypothesized that this could be attributable to the upregulation of the androgen receptor in the vastus lateralis, and that cessation of exercise and the resulting decrease in androgenic signaling was analogous to the castration in the transgenic rats, thus causing dendritic atrophy (this is discussed in greater detail in Chapter 7). This phenomenon of exercise-induced androgen sensitization is particularly interesting. Several lines of research have investigated the interactions between exercise and hormones during times of particular hormone sensitivity (e.g., puberty). Gonadal hormones play well-documented roles in human puberty, including driving development of secondary sex characteristics (Rogol, 2002), proliferation and eventual sealing of the epiphyseal plate (growth plate; Perry et al., 2007; Richmond and Rogol, 2016), and as a regulatory signal within the hypothalamic-pituitary-gonadal axis (Richmond and Rogol, 2016). The role of hormones in pubertal development makes a potential sensitization of muscle tissue to hormones an interesting possibility.

There have been few reported cases of investigation into permanent changes following adolescent exercise in humans. An analysis of prepubertal and retired female gymnasts compared against age and sex-matched controls suggests that early life participation in competitive gymnastics confers lifelong increases in bone mineral density (Bass et al., 1998). A single study is clearly not sufficient evidence to suggest that pre- or peripubertal exercise definitely causes lifelong adaptive alterations across all physiological measures, but it does provide a foundation that it is possible to confer lifelong physiological adaptations, likely specific to the particular exercise. It is interesting to speculate whether exercise during adolescence is sufficient to drive permanent alterations to anabolic hormone sensitivity, either at the muscle or other tissues, and whether such peripheral adaptations extend to their innervating cells within the central nervous system. Given that this dissertation has established a relationship between exercise, hormones, and neuroprotection, permanent alterations to either neuromuscular sensitivity to hormones, muscle morphology, or spinal neural circuitry may provide

protective benefits to later neural injury or benefits to neuroplastic adaptations, such as acquisition of new motor skills.

Concluding remarks

The findings of this dissertation have demonstrated that exercise is neuroprotective to motoneuron dendrites following the death of their neighbors via a mechanism requiring androgen action at the target muscle of the innervating motoneurons. This also demonstrates that a behavioral intervention such as exercise can have modulatory effects on both peripheral and central aspects of the neuromuscular complex. Other data presented in this dissertation suggests that some of these plastic changes are transient in adulthood, and future research could investigate whether exercise at earlier critical periods (i.e., puberty) could produce permanent alterations to the neuromuscular complex. Other lines of research could also investigate whether there are sex differences in exercise-driven neuroprotection, or whether alterations to the exercise protocol affect whether exercise remains neuroprotective.

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Cory Chew
Curriculum Vitae

Education

2015 – 2020 Indiana University, Bloomington, IN

Ph.D. Neuroscience and Psychology, conferred December 2020

Advisor: Dale Sengelaub, Ph.D.

Dissertation: *The neuroprotective effects of exercise on motoneuron dendritic atrophy are driven by androgen action at target musculature*

2011 – 2015 University of Michigan, Ann Arbor, MI
B.S. Neuroscience, conferred May 2015

Research Experience

2015 – Present Graduate Research Assistant, *Sengelabs*, Indiana University

Principal Investigator: Dale Sengelaub, Ph.D.

Studying 1) the effects of sex steroid treatment on neuron-glia interactions and 2) the physiological benefits of exercise in protecting dendritic morphology in motoneuron depletion models. Training in animal surgery, histology, optical microscopy, software-based stereology and anatomical reconstruction, hormonal assays, and genetic analysis using quantitative polymerase chain reaction (qPCR).

2014 – 2015 Undergraduate Research Assistant, *Aldridge Lab – Neural Coding of Reward and Movement in the Basal Ganglia*, University of Michigan

Principal Investigator: J. Wayne Aldridge, Ph.D.

Investigated the electrophysiological role and function of the basal ganglia in food reward processing and motion generation. Constructed and assisted in implantation of tetrode recording electrodes, handled and cared for laboratory rats, coded and analyzed video-recorded behavioral data, and conducted histological analysis of harvested neural tissue.

2013 – 2015 Undergraduate Research Assistant, *Emotion and Self-Control Lab*, University of Michigan

Principal Investigator: Ethan Kross, Ph.D.

Investigated 1) the effects of social media use and current events on the generation and regulation of negative emotions and 2) the use of novel self-distancing cognitive techniques to more effectively process negative emotions. Helped design and develop

experimental protocols, participant recruitment advertisements, and data coding protocols. Organized undergraduate lab agendas, ran human participants through experimental tasks, and performed data analysis.

Teaching Experience

2020, 2019, 2016 — Teaching Assistant, *Introduction to Neuroscience*, Indiana University

Instructor: Dale Sengelaub, Ph.D.

Helped instruct a survey course introducing undergraduates to neuroanatomy, neurobiology, sensory systems, learning and memory, and neural development. Graded exams and held regular office hours.

2020, 2018 — Associate Instructor, *Statistical Techniques*, Indiana University

Instructor: Rick Hullinger, Ph.D.

Supervised and led course discussions in lab sections for undergraduate statistics. Graded exams, course assignments, and designed review sessions.

2019 — Teaching Assistant, *Neurobiology of Neurodegenerative Diseases*, Indiana University

Instructor: Joseph Farley, Ph.D.

Instructed students in an advanced elective course that covered the molecular and systemic mechanisms underlying neurodegenerative diseases. Evaluated all coursework and developed course materials, including guest lectures and grant writing sessions.

2018 — Teaching Assistant, *Neurobiology of Learning and Memory*, Indiana University

Instructor: Joseph Farley, Ph.D.

Instructed students in an advanced elective course that covered the molecular mechanisms of synapse neuroplasticity, memory circuitry and neuroanatomy, and paradigms of behavioral learning. Developed course materials (e.g., quizzes, exams), provided feedback on all student submissions, and led guest lectures and review sessions. Advised students in one-on-one study sessions and writing workshops.

2017 — Associate Instructor, Methods of Experimental Psychology, Indiana University

Instructor: Rick Hullinger, Ph.D.

Taught the core components of experimental design, literature review, scientific writing, statistics and data analysis, and rigorous critical thinking for psychological research questions. Developed course materials, including rubrics, weekly homework

assignments, lectures, paper prompts, and grading rubrics. Graded research papers and course assignments.

Teaching Assistant, *Science of Human Sexuality*, Indiana University

Instructor: Julia Heiman, Ph.D.

Instructed students in a survey course that covered anatomy, behaviors, psychopathologies, and economics in relation to human sexuality. Developed and evaluated course materials, including exams, quizzes, interactive student polls, and guest lectures. Held regular office hours and review sessions.

2016 Teaching Assistant, *Lab in Behavioral Neuroscience*, Indiana University

Instructor: Ehren Newman, Ph.D.

Assisted in the design and instruction of a laboratory course introducing undergraduates to behavioral neuroscience techniques. Assisted in designing student experiments, teaching students how to conduct a literature review, implant subcortical recording electrodes and pharmacological infusion cannula, conduct aseptic surgical techniques, run behavioral trials, and graded student laboratory reports.

Guest Lectures

2019 "Studying Sex Using Neuroscience" – *Human Sexuality Research*

2018 "Long Term Depression" – *Neurobiology of Learning and Memory*

Publications

Chew, C., & Sengelaub, D.R. *(in press)*. Exercise is neuroprotective on the morphology of somatic motoneurons following the death of neighboring motoneurons via androgen action at the target muscle.

Chew, C. & Sengelaub, D.R. (2020). Exercise promotes recovery after motoneuron injury via hormonal mechanisms. *Neural Regenerative Research*, 15(8):1373-1376.

Chew, C. & Sengelaub, D. R. (2019). Neuroprotective effects of exercise on the morphology of somatic motoneurons following the death of neighboring motoneurons. *Neurorehabilitation and Neural Repair*, 33(8), 656-667.

Chew, C., Kiley, B. J., & Sengelaub, D. R. (2019). Neuroprotective effects on the morphology of somatic motoneurons following the death of neighboring motoneurons: A role for microglia*? Developmental Neurobiology*, 79(2), 131-154.

Cai, Y., **Chew, C.**, Muñoz, F., & Sengelaub, D. R. (2017). Neuroprotective effects of testosterone metabolites and dependency on receptor action on the morphology of somatic motoneurons following the death of neighboring motoneurons. *Developmental Neurobiology*, 77(6), 691-707.

Poster Presentations

Chew, C. & Sengelaub, D. R. (2019). Exercise is neuroprotective to motoneuron dendrites following partial motoneuron depletion via androgen action at the target muscle. *Society for Neuroscience*, Chicago, IL.

Chew, C. & Sengelaub, D. R. (2019). Run for your dendrites: Exercise is neuroprotective to motoneuron dendrites after injury. *Animal Behavior Conference*, Bloomington, IN.

Chew, C. & Sengelaub, D. R. (2018). Exercise is neuroprotective to motoneuron dendrites following partial motoneuron depletion. *Society for Neuroscience*, San Diego, CA.

Chew, C. & Sengelaub, D. R. (2016). Testosterone treatment influences quadriceps motoneuron expression of CX3CL1 following motoneuron depletion. *Indiana University Department of Psychological and Brain Sciences Graduate Research Symposium*, Bloomington, IN.

Professional Service/Experience

2019 — Committee Member, *Indiana University Committee for Fee Review*

Served as audience to a presentation given by leadership of each organization requesting funds, in which they open their financial records and justify the requested funding amount. Provided recommendations to the higher administration of Indiana University for how over $27.6 million from student tuition fees should be allocated to various university organizations and university-recognized student groups/organizations.

2018 – 2019 — Executive Committee Awards Officer, *Indiana University Graduate and Professional Student Government (GPSG)*

Managed committee meeting minutes, recruitment and management of reviewers of applications for the $40,000 in awards distributed by the IU GPSG, and communication with the constituents of the GPSG concerning awards/funding and related resources. Served on the GPSG Executive Committee, who oversee the creation and implementation of programs, benefits, and outreach intended for the benefit of the constituents of the GPSG.

2019 – 2020 — Representative, *Graduate and Professional Student Government*

2018 – 2020 — Volunteer Grant Reviewer, *Graduate and Professional Student Government*

2019 — Volunteer, *Brain Awareness Week, University Elementary*

2017	Volunteer, *Grandview Science Night*
2016	Volunteer, *Indiana University Science Fest*
2012 – 2013	Volunteer, *University of Michigan Health System*

Awards, Fellowships, and Grants Awarded

2020, 2019, 2017	Harlan Scholarship Award – $5000
2019, 2018	Indiana University Program in Neuroscience Travel Award - $900
2019	Outstanding Graduate Teaching Assistant – Department of Psychological and Brain Sciences, Indiana University
2018	Graduate Professional and Student Government Officer Fellowship – $1000

Professional Memberships and Societies

2015 – Present	Society for Neuroscience
2015 – Present	*Grey Matters*, Indiana University graduate student neuroscience club focusing on developing ideas and professional development
2018 – 2020	Indiana University Graduate and Professional Student Government